

The Effects of Air Pollution on Vascular Thrombosis

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Declaration

I hereby declare that the data published in this thesis are the result of my own work, carried out under the supervision of Dr. Patrick W. F. Hadoke, Prof. Kenneth Donaldson and Dr. Mark M. Miller at the University of Edinburgh, and that this thesis has been completed entirely by myself and has not previously been submitted for any other degree or qualification.

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Abstract

Increases in air pollution, especially the particulate component, are associated with increased cardiovascular mortality, possibly through increases in thrombogenic mechanisms. The research presented in this thesis addresses the hypothesis that diesel exhaust particulates (DEP) increase thrombogenicity by impairing the release of tissue plasminogen activator (t-PA) from vascular endothelial cells, thus inhibiting the endogenous fibrinolytic mechanisms that promote thrombus breakdown.

The initial aims of this work were to develop an *in vivo* model of thrombosis, to determine whether exposure to DEP did alter clotting. Initial attempts to develop the Folts' model (which stimulates thrombus formation via arterial stenosis and mechanical injury), first in male C57/Bl6 mice and later in male Wistar rats, were unsuccessful. An alternative approach, using ferric chloride (FeCl_3) to induce chemical injury to the rat carotid artery was found to produce reliable and reproducible thrombotic occlusion: this model was used for all subsequent experiments.

The effects of DEP on thrombus formation were assessed *in vivo* by applying the FeCl_3 model. DEP were administered via intratracheal instillation or tail vein injection 2, 6 or 24 hours prior to induction of thrombosis. The effects of DEP were compared with vehicle and suitable controls: carbon black (a clean carbon nanoparticle); quartz (a large non-carbon particle that causes well-characterised pulmonary inflammation). The time to thrombotic occlusion was significantly reduced 6h after intra-pulmonary instillation of DEP (0.5ml of a 1mg/ml suspension). In contrast, instillation of carbon black or quartz had no significant effect on thrombosis, despite causing greater pulmonary (increased neutrophils and levels of interleukin-6, tumour necrosis factor- α and C-reactive protein in bronchoalveolar lavage fluid) and systemic (C-reactive protein in plasma) inflammation than DEP. Direct administration of DEP (0.5mg/kg) to the blood stream resulted in an acute (2 hours after injection) increase in time to thrombotic

occlusion in the absence of pulmonary inflammation. A similar (but less pronounced) effect was observed following administration of carbon black (0.5mg/kg). These data suggest that the DEP-mediated increase in thrombosis is independent of pulmonary and systemic inflammation. The mechanisms involved were addressed by measuring platelet-monocyte interactions (flow cytometry) and markers of the endogenous fibrinolytic system (ELISA). Exposure (either instillation or injection) to DEP significantly increased platelet-monocyte aggregation. Carbon black and quartz produced no such effect (but did increase platelet-platelet aggregation). t-PA antigen and activity were reduced, whilst PAI-1 and fibrinogen were increased, following either instillation or injection of DEP.

The final aim was to develop a suitable dispersant for use in cell culture to determine whether DEP alter the expression (real-time polymerase chain reaction; rtPCR) and generation (enzyme-linked immunosorbent assay; ELISA) of t-PA and plasminogen activator inhibitor (PAI-1). Cell culture medium containing bovine serum albumin (0.5mg/ml; BSA) provided the best combination for DEP dispersal and maintenance of small particle size (<200nm), without detrimental effects on human umbilical endothelial cells (HUVECs). Exposure (6 and 24 hours) of HUVECs to DEP resulted in reduced basal and thrombin stimulated t-PA and PAI-1 expression. This was mirrored by reduced detection of t-PA and PAI-1 in culture medium.

In conclusion, these investigations confirm that exposure to DEP is capable of increasing the rate of thrombus formation and that this is, in part, mediated by an alteration in the endogenous fibrinolytic system. These changes did not appear to be secondary to pulmonary or systemic inflammation. Whilst cell culture experiments suggested DEP could directly alter endogenous fibrinolytic activity in endothelial cells, there was no evidence from these experiments of DEP translocation into the systemic circulation. Thus, this work suggests that DEP is capable of increasing thrombus formation *in vivo* via several mechanisms. Similar changes may account for the increased thrombus formation in humans exposed to diesel exhaust in air pollution.

Presentations

Oral:

Pollution Group Meeting, Maastricht - 11th and 12th January 2007

Vascular Group Meeting, Edinburgh - 20th February 2007

Endocrinology Group Meeting, Edinburgh - 12th July 2007

Edinburgh Pollution Group Meeting, Edinburgh - 29th August 2007

Pollution Group Meeting, Edinburgh - 27th and 28th September 2007

QMRI Open Day, Edinburgh - 25th October 2007

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BHF International Meeting, Edinburgh - 26th - 28th August 2008

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CVS Symposium Day, QMRI, Edinburgh - 2nd June 2006

BHF Site Visit, QMRI, Edinburgh - 14th January 2008

CVS Symposium Day, QMRI, Edinburgh - 4th September 2008

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Papers

Diesel Particulate-Exposed Macrophages Cause Marked Endothelial Cell Activation.

Catherine A. Shaw, Sarah Robertson, Mark R. Miller, Rodger Duffin, Caroline M. Tabor, Ken Donaldson, David E. Newby & Patrick W.F. Hadoke.
American Journal of Respiratory Cell and Molecular Biology, 2010.

Diesel Exhaust Particulate Induced Acceleration of Thrombus Formation: Role of Inflammation, Platelet Activation and Direct Interaction with the Endogenous Fibrinolytic System.

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Abbreviations

Abbreviation	Definition
ADP	Adenosine Diphosphate
AMP	Adenosine Monophosphate
ANOVA	Analysis of Variance
BAL	Bronchoalveolar Lavage
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumen
CAPs	Concentrated Ambient Particles
CB	Carbon Black
CINC-1	Cytokine-Induced Neutrophil Chemoattractant-1
COX-2	Cyclooxygenase-2
CRP	C Reactive Protein
DEP	Diesel Exhaust Particulate
DQ12	Quartz Particulate
EGM-2	Endothelial Cell Culture Medium
ELISA	Enzyme Linked Immunosorbant Assay
FITC	Fluorescein Isothiocyanate
FS	Forward Scatter (Flow Cytometry)
H ₂ O	Water
H ₂ O ₂	Hydrogen Peroxide
ICAM-1	Intercellular Adhesion Molecule-1
IL-6	Interleukin 6
LDH	Lactate Dehydrogenase
LPS	Lipopolysaccharide
MIP-2	Macrophage Inflammatory Protein-2
NIST	National Institute of Standards and Technology
NO	Nitric Oxide

oxyLDL	Oxidised Low-Density Lipoprotein
PAI-1	Plasminogen Activator Inhibitor
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PF-3	Platelet Factor 3
SEM	Standard Error of Mean
SFM	Serum Free Endothelial Cell Culture Medium
SS	Side Scatter (Flow Cytometry)
TE	Tris EDTA Buffer Solution
TGF- β 1	Transforming Growth Factor Beta-1
TNF α	Tumour Necrosis Factor alpha
t-PA	Tissue Plasminogen Activator
UFPs	Ultrafine Particles
u-PA	Urokinase Plasminogen Activator

Chapter 1

Introduction

Cardiovascular disease is one of the most significant causes of morbidity and mortality in the developed world. Many risk factors are recognised in the development of cardiovascular disease, such as age, sex, heredity, smoking, weight, physical inactivity, high cholesterol and high blood pressure. A growing body of epidemiological evidence now supports the theory that exposure to environmental air pollution also increases the risk of cardiovascular events, although it is not clear which biological mechanisms, or which components of air pollution, are responsible for these effects. The number of premature deaths attributed to air pollution worldwide is estimated to be ~2 million per annum (World Health Organisation 2002, World Health Report, Geneva, <http://www.who.int/whr/2002/en>). Indeed, the significance attributed to the association between air pollution and cardiovascular disease is illustrated by the decision, in 2004, of the American Heart Association to publish a scientific statement on this subject (Brook *et al.* 2004). This has recently been updated, with the emphasis changed to address the relationship between cardiovascular disease and *particulate matter* air pollution; based on increasing evidence that even relatively brief exposures to particulate matter with a diameter $<2.5\mu\text{m}$ ($\text{PM}_{2.5}$) can cause increased fatal and non-fatal cardiovascular events (Brook *et al.* 2010). The continuing impact of this problem has recently been emphasised (and is increasingly brought to the public's attention) by stories in national and international media; such as the problems of acute smog caused by peat fires and unusually high temperatures in Moscow this summer (<http://articles.latimes.com/2010/jul/29/world/la-fg-moscow-smog-fires-20100729>). Also of particular significance is the problem that atmospheric pollution is causing China (<http://news.bbc.co.uk/1/hi/world/asia-pacific/6265098.stm>) and her neighbours (<http://www.timesonline.co.uk/tol/news/world/asia/article7072220.ece>). The work described in this thesis addresses the hypothesis that exposure to particulate matter air pollution (specifically ultrafine, diesel combustion-derived particles) increases the risk of cardiovascular events by increasing the chance of vascular thrombosis. Finding results to support this theory may provide an opportunity to develop new treatments or preventative measures to lessen the effects of air pollution on the cardiovascular system.

1.1 Atmospheric Pollution

1.1.1 Historical Evidence for the Adverse Effects of Environmental Pollution on Human Health

A growing body of evidence, accumulated over more than 50 years, has shown that exposure to air pollution has adverse effects on human health. The growing realisation that air pollution could have a significant, and acute, impact on human morbidity and mortality received corroboration from a number of dramatic incidents in the first half to the 20th century (see Simkhovich *et al.* 2008). Although preceded by disasters such as the Meuse Valley smog (Nemery *et al.* 2001) and the industrial pollution incident at Donora (Pennsylvania, USA; Helfand *et al.* 2001), the most notable occurrence from a British perspective must be the London smog of December 1952. This was caused by high levels of particulate air pollution, mostly derived from burning coal, combined with a period of cold weather and anticyclone (windless) conditions, which prevented particulate matter from being cleared from the air (Davis *et al.* 2002; Dooley *et al.* 2002; Hunt *et al.* 2003). In the weeks following the London smog, it was estimated that ~4,000 people died as a result, and a further 100,000 people became ill with respiratory problems. However, more recent work has estimated that the number of deaths resulting from the smog was much higher, at around 12,000 (Bell *et al.* 2001). This cluster of severe incidents (the London smog is widely regarded as the worst air pollution incident in the United Kingdom) had a great effect on stimulating environmental research and promoting public awareness of the relationship between air pollution and health. It also resulted in legislation aimed at reducing the extent and toxicity of environmental pollution, leading to the introduction of regulations such as the Clean Air Act of 1956 (and the Clean Air Act (1963) and the Air Quality Act (1967) in the USA). Introduction of these measures has been central to a reduction in UK particulate air pollution; from $130\mu\text{g}/\text{m}^3$ to around $20\mu\text{g}/\text{m}^3$ 50 years later (<http://www.airquality.co.uk>). Despite this improvement, atmospheric pollution remains a considerable Public Health issue (Ware. 2000; McCreanor *et al.* 2007) as emphasised in a recent report from the UK Parliament (<http://www.publications.parliament.uk/pa/cm200910/cmselect/cmenvau/d/229/229i.pdf>). Indeed, the association between air pollution and mortality does not

seem to have a demonstrable threshold and remains evident below current national and international standards for air quality (Ware. 2000). Furthermore, the gravimetric measure used to demonstrate the reduction in particulate pollution hides the increase in combustion-derived *nanoparticles*, which has paralleled the increase in road vehicles in the UK over the same period. Due to their small size, nanoparticles contribute very little to mass-based measures of particulate pollution, but are much more likely to have effects on health than larger particles (see Mills *et al.* 2009).

1.1.2 Components of Atmospheric Pollution

Air pollution comprises a complex mixture of gases, liquids and particles (Table 1.1) that will vary in different locations, at different times and under different weather conditions (Harrison & Yin. 2000; Simkhovich *et al.* 2008; Brook *et al.* 2010). These components are continually changing and also altering through chemical reactions with each other and with natural gases in the atmosphere. Some components are essentially harmless (eg sodium chloride) whilst others are not particularly toxic at ambient levels. However, certain components can have individual toxic effects and can also act in synergy or antagonistically with other constituents of the pollutant cocktail.

Atmospheric pollutants arise from the natural environment (eg. Wildfires, such as the peat fires that recently caused such problems in Moscow) and from human activities (industry, agriculture, traffic, power generation, biomass burning) (Simkhovich *et al.*, 2008; Brook *et al.*, 2010). In addition to these primary pollutants, secondary pollutants can arise as a consequence of chemical reactions (often involving sunlight and water vapour) in the atmosphere. For example, ozone is largely generated as the product of chemical reactions occurring when exhaust pollution is exposed to sunlight (Joseph. 2007).

Table 1.1 - The Major Components of Atmospheric Pollution.

D_{ae} , aerodynamic diameter. Adapted from Simkhovich *et al.* 2008 and Brook *et al.* 2010.

Particulate Matter (PM)	Coarse (D_{ae} 2.5-10 μ m)
	Fine (D_{ae} <2.5 μ m)
	Ultrafine (D_{ae} <0.1 μ m)
Gases	Carbon monoxide (CO)
	Nitrogen oxides (NO, NO ₂)
	Ozone (O ₃)
	Sulphur dioxide (SO ₂)
	Carbon dioxide (CO ₂)
	Carbon monoxide (CO)
Volatile and Semi-Volatile Organic Compounds (VOC/ SVOC)	Benzene
	Toluene
	Xylene
	1,3-butadiene
	Polycyclic Aromatic Hydrocarbons

1.1.2.1 Particulate Matter

Given the complex and dynamic nature of air pollution, it is difficult to attribute its effects on health to a single constituent. Over the past decade, however, increasing emphasis has been placed on the role of particulate matter, and in particular on the fine and ultrafine particles. Even so, it is important to remember that these particles only account for ~2% of pollutant mass (with the remaining 98% attributable to gases and organic vapour phase compounds; Brook *et al.* 2010). The National Atmospheric Emissions Inventory monitors particulate air pollution at 120 sites throughout the UK. As expected, emissions are highest in urban areas and along major roads. Average UK levels meet the government standards of $50\mu\text{g}/\text{m}^3$ of sampled air (<http://www.airquality.co.uk/standards.php>). However, standard levels are regularly exceeded, with particulate levels in London often reaching $300\mu\text{g}/\text{m}^3$ (<http://www.airquality.co.uk/standards.php>).

Particulate matter can be derived from natural (e.g. windblown dust, wildfires) and human (eg road and agricultural dust, tyre wear emissions, wood combustion, combustion, demolition, mining) activities (Simkhovich *et al.* 2008; Harrison & Yin. 2000). These ambient particles can comprise inorganic compounds (sulphates, nitrates, chloride, ammonia), trace metals, crystals, biological components (pollens, spores, bacteria) and adsorbed volatile/semivolatile organic compounds. Environmental particulate air pollution is measured by a global sampling convention and categorised based on size (see Table 1.1); coarse particles (PM_{10}) range from $2.5\text{-}10\mu\text{m}$ in diameter, fine particles ($\text{PM}_{2.5}$) range from $100\text{nm-}2.5\mu\text{m}$, and ultrafines are smaller than 100nm . Coarse particles are able to pass the larynx and reach the upper airways, fine particles are able to penetrate the unciliated regions of the lungs, but only ultrafine particles (UFPs) are able to travel deep into the lungs, finally depositing in the alveoli (Donaldson *et al.* 2001). It should be noted, however, that, whilst most studies that link particulate matter to cardiovascular disease focus on particle mass, this may not be the best measure of the activity of these pollutants (Simkhovich *et al.* 2008). Some of the effects of particulates are also undoubtedly due to their chemical composition and other characteristics (eg, solubility, charge,

surface area, pulmonary deposition, stability in the atmosphere and in biological tissue). Evidence now suggests that particle size and surface area are important contributors to the adverse effects associated with PM₁₀ exposure, as UFPs cause a greater pulmonary inflammatory response than fine particles made of the same materials. The toxicity of particulate matter is, therefore, usually considered to be related to the particle number, size, chemical composition and surface area. In addition to their carbon core, combustion-derived particulates often have associated toxic compounds (eg, transition metals, organic compounds, semiquinones, endotoxins) adsorbed to their surface (Scheepers & Bos. 1992; Donaldson *et al.* 2005).

Given the complexity of atmospheric pollution it has been suggested that investigations should focus on the sources of pollutants that are of the greatest concern to human health, rather than try to document singly which constituents are most toxic (Brook *et al.* 2010). Of particular concern in recent years has been the significance of atmospheric pollutants released from road transport. Many individuals, particularly in poor areas, live close enough to roads (within 500m) to be consistently exposed to high levels of traffic exhaust fumes. In addition, modern lifestyles dictate that a significant number of people spend a considerable proportion of their day travelling in motor vehicles (Solomon *et al.* 2008). Certain occupations (eg, traffic wardens, traffic police) are more susceptible to the effects of air pollution, due to their increased exposure. The work described in this thesis focuses, therefore, on diesel combustion, which contributes a significant component of vehicle emissions and produces a complex mixture of potentially toxic gases and particles.

Ultrafine particles make up the majority of all diesel exhaust particles, with up to 50% by mass of urban PM₁₀ being made up of ultrafine carbon-centred particles (Donaldson *et al.* 2005). These particles are associated with metals (including transition metals), ammonium salts of nitrogen, sulphur and chlorine, as well as geological dust and organic matter (Donaldson *et al.* 2005). Organic compounds, polycyclic aromatic hydrocarbons and oxidized transition metals carried on the

surface of combustion-derived nanoparticulates (Scheepers & Bos. 1992) can generate oxidative stress and inflammation (Donaldson *et al.* 2005).

1.1.3 Epidemiological Evidence for the Adverse Health Effects of Atmospheric Pollution

There is now considerable evidence in the scientific literature (confirming the observations from dramatic pollution episodes in the 20th century) showing emphatically that exposure to atmospheric pollution increases morbidity and mortality (Pope & Dockery. 2006). Indeed, despite reductions of atmospheric air pollution in many locations following the introduction of environmental legislation, the World Health Organisation (WHO) currently estimates that ~2 million people die each year due to conditions caused by exposure to air pollution. This represents 3.6% of the 55 million deaths occurring worldwide each year (WHO Statistics: <http://www.who.int/mediacentre/factsheets/fs313/en/index.html>). These deaths arise from both acute and chronic exposures to atmospheric pollution, largely from cardiovascular disease, respiratory disorders (chronic obstructive pulmonary disease, pneumonia) and cancer (largely lung cancer) (Simkhovich *et al.* 2008).

Many epidemiological studies have shown an association between increased air pollution and increases in cardiopulmonary morbidity and mortality, and these have been reviewed extensively (Pope & Dockery. 2006; Simkhovich *et al.* 2008; Brook *et al.* 2010). Large-scale studies have been conducted in order to address concerns about city selection bias; one of the largest was the National Morbidity, Mortality and Air Pollution Study (NMMAPS), conducted in the U.S. Several reports have been published using this data, including Samet *et al.* (2000), which assessed the effects of PM₁₀ in 20 major U.S. cities over a period of 8 years and reported that levels of fine particulate air pollution were associated with increased deaths from cardiovascular and respiratory illnesses. Dominici *et al.* (2003) suggested that a 10µg/m³ elevation in PM₁₀ results in a 1.87% increase in cardiopulmonary mortality, whereas a 10µg/m³ elevation in PM_{2.5} results in a 6% increase in cardiopulmonary

mortality (Pope *et al.* 2002). This suggests that the fine and ultrafine fractions of particulate pollution are most likely to cause acute cardiovascular and pulmonary problems although it is possible these differences are due to the source of the particulates used in the two studies. Similar studies were also conducted in Europe; Air Pollution and Health: A European Approach (APHEA and APHEA-2) examined daily PM and related mortality in multiple cities (Katsouyanni. 2003; Analitis *et al.* 2006) and found that increases in cardiovascular and respiratory mortality were significantly associated with increases in PM air pollution. Similar increases in cardiovascular mortality were reported following short-term exposure to PM in China (Kan *et al.* 2003; Qian *et al.* 2008; Wong *et al.* 2008a) and Bangkok (Wong *et al.* 2008b). In an attempt to compare data for different continents, the Air Pollution and Health: A Combined European and North American Approach (APHENA) study analysed data from the APHEA, NMMAPS and Canadian studies (Samoli *et al.* 2008). This study found that all-cause mortality increased between 0.2% and 0.6% for a $10\mu\text{g}/\text{m}^3$ increase in daily ambient PM_{10} . The greatest effects were observed in Canada, with individuals over 75 years of age being at greater risk of cardiovascular mortality. Furthermore, worldwide evidence collected in the first AHA statement (Brook *et al.* 2004) confirms that short-term elevations in $\text{PM}_{2.5}$ ($10\mu\text{g}/\text{m}^3$) result in a 0.4% to 1.0% increase in daily mortality, with particular increases observed in cardiovascular deaths.

1.1.4 Interaction of Pollutants with the Cardiovascular System

It is now apparent that the inability of many people to avoid continued long-term exposure to pollution may silently and continuously increase acute cardiovascular risk (Pope & Dockery. 2006; Simkhovich *et al.* 2008; Brook *et al.* 2010). Investigation of atherosclerotic risk factors is difficult in the general population, due to the silent progression of the condition and the number of different factors that contribute to its development. However, there is now some evidence to suggest that chronic exposure to air pollution may accelerate the development of atherosclerosis in healthy individuals with no previous signs of cardiovascular disease, but with

slightly increased low-density lipoprotein levels (Kunzli *et al.* 2005b). For example, increased PM_{2.5} has been associated with an increase in carotid artery intima-media thickness (Kunzli *et al.* 2005b). Similarly, coronary artery calcification is increased in residents living closest to main roads (Hoffmann *et al.* 2007). Both these observations are consistent with pollution causing progression of atherosclerosis. Further evidence (from the Netherlands) shows that people living close to major roads have an increased incidence of cardiopulmonary mortality (Hoek *et al.* 2002), and it has been suggested that 6% of all coronary heart disease deaths in the UK could be due to traffic-related pollution (Maheswaran *et al.* 2005).

It is also well established that PM triggers cardiovascular events that occur rapidly (within hours or days) following exposure (Simkhovich *et al.* 2008; Brook *et al.* 2010). This is consistent with the demonstration that such acute exposures can induce a variety of direct changes on various aspects of the cardiovascular system (including, heart rate, heart rate variability, blood pressure, vascular tone, blood coagulability and the incidence of cardiovascular arrhythmias). Studies in humans have shown that exposure to PM can increase blood pressure (Zanobetti *et al.* 2004; Ibaldo-Mulli *et al.* 2001), plasma viscosity (Peters *et al.* 1997b) and prothrombin time (Baccarelli *et al.* 2007) and can impair vascular reactivity (Brook *et al.* 2002; Mills *et al.* 2005; O'Neill *et al.* 2005). Studies from Edinburgh University have also shown that exposure to diesel exhaust particulate (DEP) increases *ex vivo* thrombus formation and *in vivo* platelet activation in healthy human volunteers (Lucking *et al.* 2008). Inhaled DEP has also been shown to cause vascular dysfunction and impair endogenous fibrinolysis in humans (Mills *et al.* 2005; Mills *et al.* 2007). PM exposure is also associated with altered heart rate and heart rate variability (Devlin *et al.* 2003; Pope *et al.* 2004; Gold *et al.* 2000; Pope *et al.* 1999a; Pope *et al.* 1999b); although whether these are increased (Pope *et al.* 1999b) or reduced (Gold *et al.* 2000) may be dependent on the type of particles and the nature of the exposure. These are likely to be more significant in individuals with underlying risk factors and are associated with an increased overall risk of myocardial infarction (Tsuji *et al.* 1996). This is consistent, therefore, with the demonstration that exposure to air pollution significantly increases hospitalizations due to a number of cardiovascular

diseases (Burnett *et al.* 1999; Schwartz *et al.* 1999; Hoek *et al.* 2001; Maheswaran *et al.* 2005). For example, exposure to high levels of air pollution causes a transient increase in the risk of acute myocardial infarction, which is greatest 1-2 hours after exposure and persists for up to 24 hours (Peters *et al.* 2004).

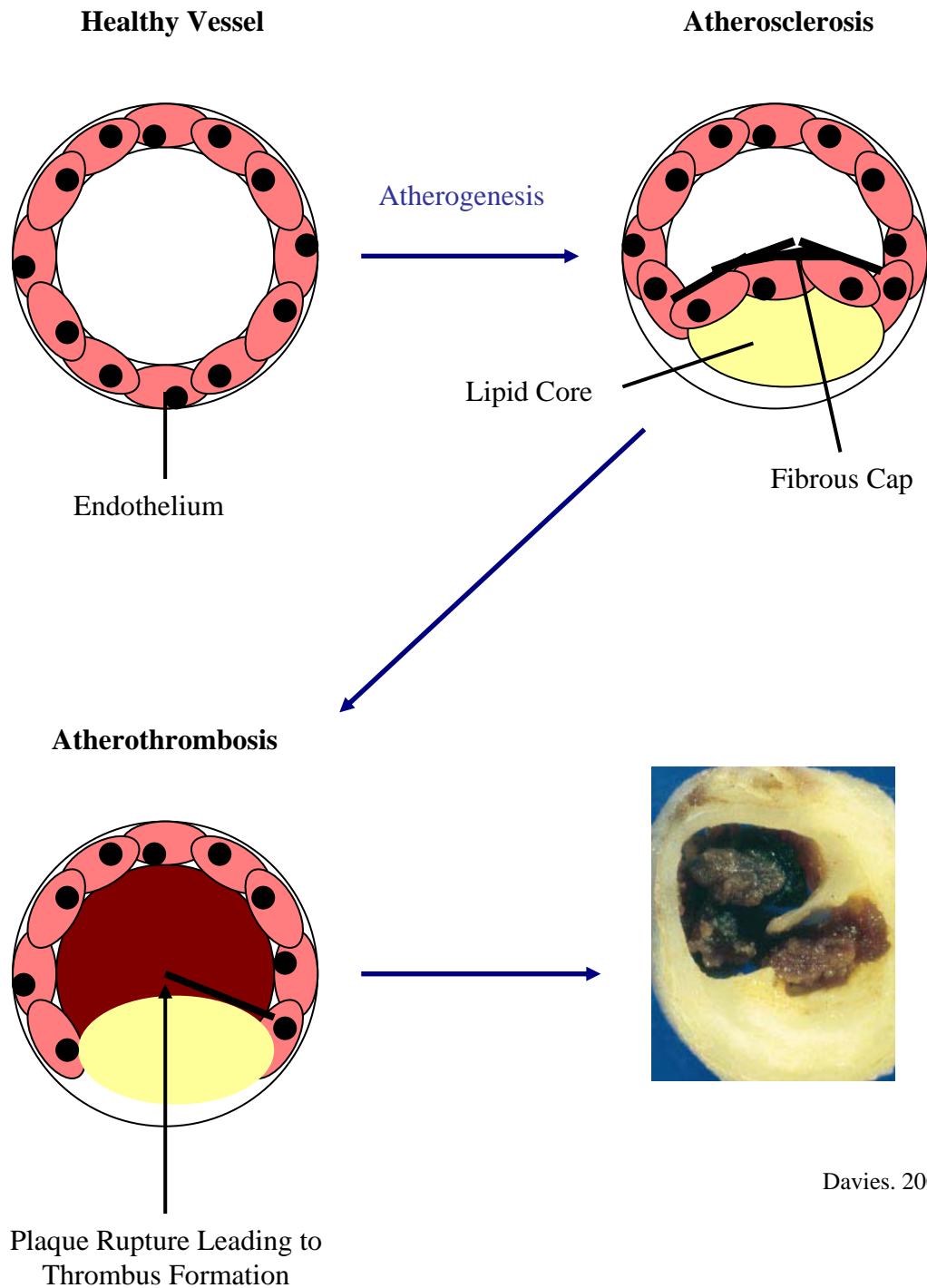
It is apparent from short- and long-term (cohort) investigations that risk estimates relative to unit exposure are consistently higher for chronic studies. The most obvious explanation for this is that short-term studies assess the acute effects of exposure whereas longer studies assess the cumulative effects of long-term exposure to pollution (Pope & Dockery. 2006). Given the considerable evidence that airborne particulate pollution has a significant impact on human cardiovascular disease, it is important to understand the underlying pathology of atherosclerosis and the thrombotic events that are often the cause of acute events in patients with this condition.

1.2 Atherothrombosis

Atherosclerosis is the leading cause of heart attack and stroke in the developed world, and has a number of well-described modifiable (including high cholesterol, high blood pressure and cigarette smoking) and non-modifiable (including heredity, sex and age) risk factors. Atherosclerosis is thought to be an inflammatory process; a wound healing response that continues after the injury is healed. It is characterised by the formation of lipid-rich plaques in medium-sized arteries, such as the carotid and coronary arteries. Advanced atherosclerotic plaques consist of a necrotic core of lipids and inflammatory cells, surrounded by a fibrous, collagen-rich cap made up of smooth muscle cells and extracellular matrix (Figure 1.1. Raines & Ross. 1993; Ross. 1993; Ross. 1999). These block the flow of blood to important tissues, leading to ischaemia and the development of the conditions associated with this disease, including angina pectoris, myocardial infarction, stroke and peripheral vascular disease (Badimon *et al.* 1993; Ross. 1993a; Ross. 1999; Fuster *et al.* 2005). Until relatively recently it was accepted that progressive narrowing of the vessel lumen,

Figure 1.1 - Schematic Representation of Atherosclerotic Plaques.

Atherosclerotic plaques form in large conduit vessels within the vessel wall. They are usually eccentric: occupying only one side of the vessel. Thrombi can form upon plaque rupture, which can cause complete vessel occlusion leading to myocardial infarction or stroke. Schematic representation of plaque rupture and occlusive thrombus formation is shown alongside a pathology photograph of an occluded vessel (adapted from Davies. 2000).



Davies. 2000

perhaps combined with vasospasm, was the primary cause of myocardial infarction and stroke, with thrombus thought to play only a minor (if any) role (see Weissberg. 2000). More recently, however, this model has been altered, with improved understanding of the role of “vulnerable” plaques, plaque rupture and acute, catastrophic occlusive thrombus formation (Fuster *et al.* 2005; Jackson. 2007). This has led to unification of several different hypotheses (relating to the roles of arterial inflammation, lipid accumulation, rupture and thrombus formation) under the umbrella term “atherothrombosis”. In order to understand the pathogenesis of atherothrombosis, it is important to understand the normal structure and function of the arterial wall.

1.2.1 Normal Vessel Structure

The structure of the vascular wall varies between arteries, veins and capillaries and can also vary in arteries from different anatomical locations. In general, arteries comprise three distinct layers; the *tunica intima*, *tunica media* and *tunica adventitia*, each of which has a distinct structural and functional role.

1.2.1.1 The Innermost Layer - The *Tunica Intima*

The *tunica intima* comprises a confluent monolayer of endothelial cells lining the luminal surface of the artery and anchored to the *tunica media* by a basement membrane. This endothelial cell layer is present in all vessel types and is the only cell layer in the capillary wall, allowing passage of substances from the blood into the tissue under the regulation of substances such as cytokines (Bucana *et al.* 1988; Horvath *et al.* 1988) histamine and bradykinin (Majno *et al.* 1969). The structure of the *tunica intima* can vary in different anatomical locations with regions of intimal thickening developing, apparently as a physiological response to mechanical stress, caused by variations in flow and/or wall tension (Ku *et al.* 1985; Stary. 1987; Stary *et al.* 1994). Intimal thickening can be described as eccentric or diffuse, although

distinction on this basis can be difficult (Stary *et al.* 1992). Eccentric intimal thickening is an abrupt and focal increase in intimal thickness, often associated with arterial bifurcations. It has been observed from the first weeks of life and shows a high degree of variation. In contrast, diffuse intimal thickening describes a less focal, often circumferential pattern around the vessel. Whilst these formations are normal, and considered to be a disease free process, they occur at locations associated with the development of advanced atherosclerotic lesions, in particular in the coronary artery (Stary. 1989).

The endothelium contributes to control of vascular tone (Furchgott & Zawadzki. 1980) by producing vasorelaxing (eg. nitric oxide (Palmer *et al.* 1987), prostacyclin, endothelium-derived hyperpolarising factor (Nagao & Vanhoutte. 1992)) and vasocontracting (eg. thromboxane (Ingberman-Wojenski *et al.* 1981), endothelin-1 (Yanagisawa *et al.* 1988)) factors. A healthy endothelium also acts as an anti-coagulant barrier, producing a number of anti-thrombotic factors, including heparin-like anti-coagulant proteoglycans (Marcum, 1986), and enzymes that degrade pro-aggregants (Cooper, 1979) or activate plasminogen (Kester, 1969). It can also restrict platelet activation by production of nitric oxide and prostacyclin (Radomski *et al.* 1987a; Radomski *et al.* 1987b).

1.2.1.2 The Middle Layer - The *Tunica Media*

The middle layer of the vessel wall is composed of sympathetically-innervated vascular smooth muscle cells interspersed with collagen, elastic fibres and the elastic laminae, which form its borders. The *tunica media* is absent from capillaries and most veins, but is a substantial component of arteries. The composition of the *tunica media* can vary greatly depending on the position of the artery; in the aorta it is rich in elastic fibres and relatively poor in smooth muscle, making the aorta capable of accommodating pulsed blood flow. Smaller 'resistance' vessels have a *tunica media* rich in vascular smooth muscle cells, which provides greater regulation of blood flow and pressure (Berne, 1985).

The main role of the *tunica media* is to regulate blood flow via control of vessel diameter, which is regulated by changes in smooth muscle tone. Vasoconstriction is mediated predominantly by changes in intracellular calcium levels in smooth muscle cells, often following direct stimulation of appropriate cell surface receptors (such as the α_1 adrenoceptor; Heusch *et al.* 1984). However, some arteries contract in response to intra-luminal pressure (myogenic tone; Bund. 2000; Brekke *et al.* 2002; Matchkov *et al.* 2002) and contraction can be induced by compounds (eg Endothelin-1) released from the endothelium (Ihling *et al.* 2001). Similarly, vasodilatation is generally caused by a reduction in intracellular calcium, often as a result of stimulation of guanylate and/or adenylate cyclases (Gruetter *et al.* 1979) or changes in cell polarisation. These can be induced by a variety of stimuli, such as shear stress (Snow *et al.* 2001) or vasoactive compounds; some acting directly on the smooth muscle cell (eg. isoprenaline (Itoh *et al.* 1985; Oriowo. 1998)) and some (eg, acetylcholine, bradykinin (Chand & Altura. 1981; Kato *et al.* 1997)) by stimulating the release of vasodilators (nitric oxide (NO), prostacyclin, endothelium-derived hyperpolarising factor) from the endothelium (Siegel *et al.* 1989; Harada *et al.* 2000; Gwozdz *et al.* 2007).

1.2.1.3 The Outermost Layer - The *Tunica Adventitia*

The *tunica adventitia* is a layer of extracellular matrix components; consisting mostly of collagen, although sympathetic nerve terminals, vascular smooth muscle cells, fibroblasts and macrophages may also be present. It lies outside the external elastic lamina, in arteries and in veins, and its main roles are to provide mechanical strength and stability to the vessel whilst anchoring it to surrounding tissue. In large vessels, the *tunica adventitia* contains microvessels (*vasa vasorum*; Clarke *et al.* 1965; McGeachie *et al.* 1982), which deliver oxygen to cells that lie beyond the range of diffusion of oxygen from luminal blood.

1.2.2 Atherogenesis

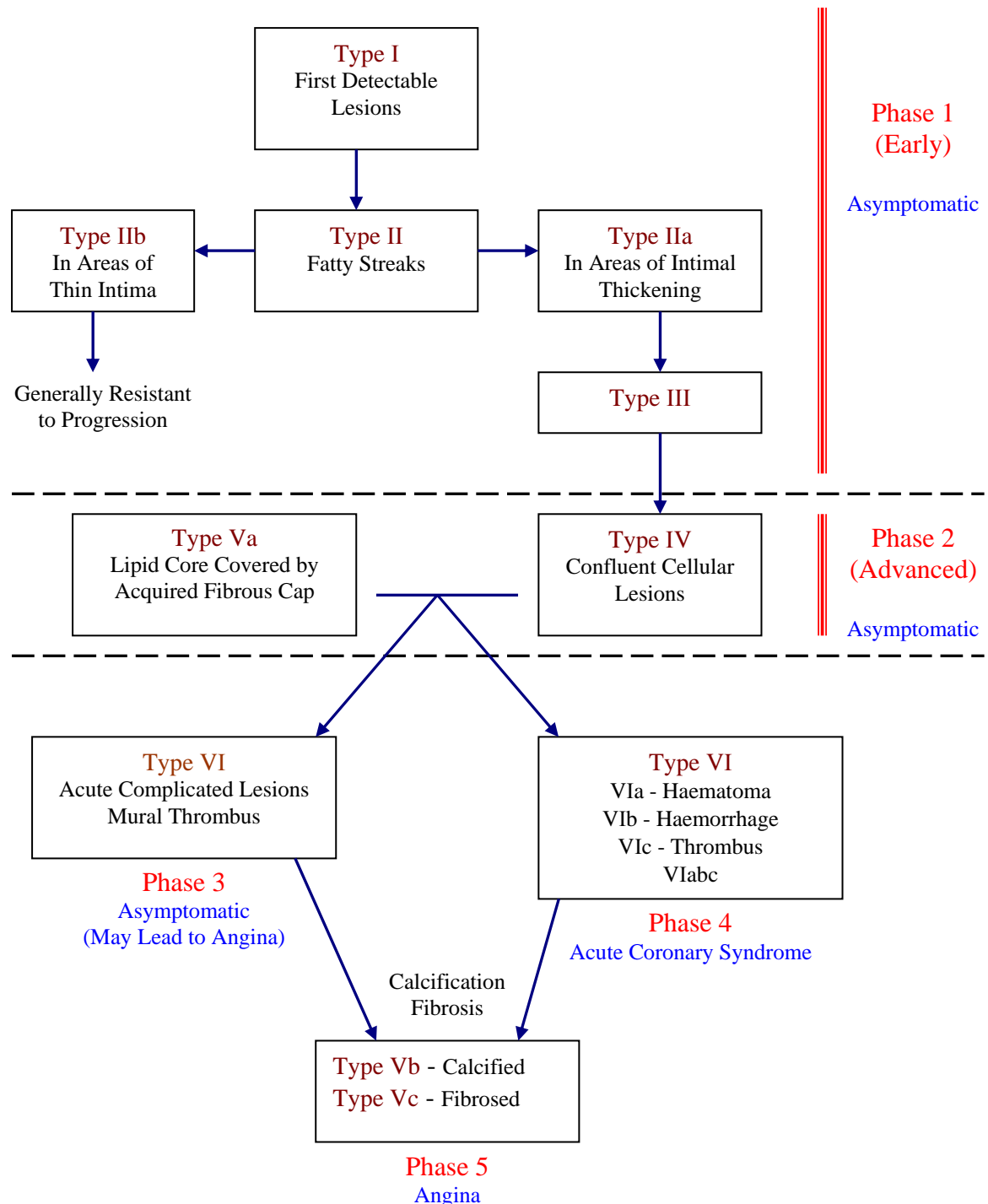
In order to understand the clinical significance of pollution-mediated increases in thrombus formation, a general appreciation of the pathogenesis of atherosclerosis is required. However, since the work described in this thesis does not address formation of atherosclerosis directly, a detailed review of atherogenesis would be inappropriate, particularly since many excellent reviews are available in the literature (including Badimon *et al.* 1993; Ross. 1993a; Ross. 1993b; Ross. 1999; Weissberg. 2000; Fuster *et al.* 2005; Rudd *et al.* 2005).

Since the pioneering work of von Rokitansky and Virchow in the 19th century (see Fuster *et al.* 2005), models for the pathogenesis of atherosclerosis have been repeatedly revised and refined (Ross & Glomsett. 1976a; (Ross & Glomsett. 1976b), leading to Ross's "Response-to-Injury" hypothesis (Ross *et al.* 1977; Ross. 1987; Newby. 2000) which itself has gone through several revisions (Ross. 1993a; Ross. 1993b; Ross. 1999). It is now generally accepted that atherosclerosis is a disorder of lipid handling and inflammation in the arterial wall, leading to its description as an inappropriate 'wound healing' response (Hansson. 2005) or a "defence mechanism gone awry" (Ross. 1993c).

Atherosclerotic lesions can be broadly sub-divided into three main types; fatty streaks, fibrous plaques and complicated lesions (Ross & Glomsett. 1976a; Ross & Glomsett. 1976b), although this is a considerable over-simplification. Based on a number of clinical and histological criteria the American Heart Association Committee on Vascular Lesions provided a basis for a more detailed lesion classification (Stary *et al.* 1994). This has been updated relatively recently (Stary *et al.* 2000) but can still be criticised for failing to identify plaque erosion or vulnerable, thin-capped fibroatheromas (Fuster *et al.* 2005). Consequently, an alternative classification has been proposed that takes account of these categories (Virmani *et al.* 2000). For simplicity, and a level of detail appropriate for this thesis, lesion development and its clinical consequences are probably best summarised in a figure (Figure 1.2).

Figure 1.2 - Classification of Atherosclerotic Lesion Development and its Clinical Consequences.

Phase 1 lesions (Types I-III) are common and often found in young people. Phase 2 (Type IV and Va) lesions do not necessarily cause stenosis but may be prone to rupture due to increased inflammation, their high lipid content and thin fibrous cap. These lesions can develop into those associated with acute Phases 3 and 4. In Phase 3, acute, complicated Type VI lesions develop from ruptured or eroded Type IV and Va lesions. This leads to the (often asymptomatic) development of mural (non-occluding) thrombus and can occasionally cause angina. In Phase 5, clinical symptoms become evident as fixed or repeated occlusive thrombus forms on complicated Type VI lesions. Calcification or fibrosis produces type Vb and Vc (Phase 5), which may cause angina. Adapted from Corti *et al.* 2004; Fuster *et al.* 2005.



1.2.2.1 Early Atherosclerotic Lesion Development

The lesions associated with the early stages (Phase 1) of atherosclerosis can be divided into 3 types: types I, II and III. Types I and II are found predominantly in children, although they can be identified in adults at sites in the vasculature not normally prone to the development of advanced lesions. Type I lesions consist of the first detectable deposits of lipid in the *tunica intima* and contain foam cells (macrophages containing phagocytosed lipid droplets (Stary. 1987)). Type II lesions are characterised by the formation of visible yellow fatty streaks/patches that can also be identified by staining neutral lipid (for example with Oil Red O or Sudan IV (Uemura *et al.* 1964)). They comprise foam cells formed into organised layers (Stary *et al.* 1994) with small amounts of extracellular lipid (mainly cholesterol esters, cholesterol and phospholipids (Katz *et al.* 1976)). Type II lesions can develop in arterial locations predisposed to develop intimal thickening (Type IIa; which are more likely to progress into atherosclerotic lesions) or in areas where the intima is thin and contains few smooth muscle cells (Type IIb; which are resistant to progression and only develop into advanced lesions in individuals with high circulating levels of atherogenic lipoproteins (Stary *et al.* 1994)). Type III lesions form a morphological and chemical bridge between type II and Type IV (atheroma) lesions. Type III lesions contain extracellular lipid droplets that form pools that lie below the layer of macrophages and foam cells and above the smooth muscle cell layer, disrupting smooth muscle cell structure (Stary *et al.* 1994). They contain much more free cholesterol, fatty acids and triglycerides than type II lesions.

1.2.2.2 Development of Advanced Atherosclerotic Lesions

Late atherosclerotic lesion development can be divided into a further 4 phases (Figure 1.2) incorporating type IV, V and VI lesions. Phase 2 involves development of type IV and type Va lesions (atheroma) which comprise large accumulations of extracellular lipid (the lipid core), inside an extensive but well-defined intimal area.

Type Va lesions (*fibroatheroma*) are those in which fibrous tissue grows over a pre-existing lipid core. Lipid cores thicken the artery wall and are generally visible to the naked eye but these lesions do not always cause narrowing of the vascular lumen, as they can be offset by outward remodelling of the affected artery (Glagov *et al.* 1987). Type IV lesions have great potential clinical significance as the characteristic lesion of advancing atherosclerosis, even though they generally remain asymptomatic. In Phase 3, acute, complicated type VI lesions develop following rupture or erosion of existing (type IV or Va) plaques. This causes the development of mural (non-obstructive) thrombosis, which, while usually clinically-silent, can cause angina. Alternatively, type IV and Va lesions can rupture causing fixed or repetitive occlusive thrombosis (Phase 4). This produces type VI, or *complex*, lesions. These are defined as type IV or V lesions with one or more of the following superimposed features: haematoma (type VIa), haemorrhage (type VIb) and thrombosis (type VIc). Lesions that exhibit all three of these features are classified as type VIabc (Stary *et al.* 1994). These processes produce clear clinical symptoms in the form of acute coronary syndrome (although they can also be asymptomatic), with morbidity and mortality from atherosclerosis largely due to rupture of type IV and V lesions. Finally, calcification or fibrosis of advanced lesions (Phase 5) can produce type Vb and Vc lesions, respectively, which may obstruct blood flow and cause angina (Stary *et al.* 1994).

Lesion rupture can vary greatly in extent and severity. Small ulcerations, often consisting of a loss of the endothelial cell layer from the surface of the lesion and only visible on microscopic examination, may occur. Alternatively, deep fissures can expose and release the highly thrombogenic lipid core, causing thrombus formation and complete arterial occlusion. As described (above), Type IV and Va lesions are particularly prone to fissuring and subsequent thrombus formation (Davies & Thomas. 1985). Many factors may contribute to the vulnerability of these lesions, including the presence of inflammatory cells in the lesions (Tracy *et al.* 1985), the release of enzymes and cytotoxic substances by macrophages within the lesions leading to breakdown of the fibrous cap (Steinberg & Witztum. 1990; Henney *et al.*

1991), coronary spasm (Nobuyoshi *et al.* 1991), shear stress (Ku *et al.* 1985) and structural weaknesses due to lesion composition (Richardson *et al.* 1989). There is evidence to suggest that fissures are more likely to occur in “shoulder regions” of lesions, which have accumulations of foam cells and relatively thin fibrous caps (Davies *et al.* 1993). Fissures may also be able to reseal, incorporating thrombi into the lesion through the formation of a new fibrous cap (Davies *et al.* 1985; Falk. 1992).

1.2.2.3 Initiation of Lesion Formation

The processes leading to the formation of type I lesions remain unclear. The ‘Response-to-Injury’ hypothesis suggests that damage (often non-denuding damage) to the endothelium causes intimal thickening by stimulating smooth muscle cells to migrate from the *tunica media* to the *tunica intima*, where they then proliferate (Ross & Glomset. 1976). The nature of the initial insult remains controversial but there is certainly evidence to suggest that plasma-derived lipoproteins accumulate in the arterial intima, causing specific cellular responses essential to the initiation of lesion development (Getz. 1990). Another theory suggests that platelets and/or fibrin deposits on the intimal surface could be the initial event in atherosclerosis (More *et al.* 1957; McMillan. 1965; Spurlock & Chandler. 1987). Many *in vivo* studies have been conducted in order to study the processes involved, such as artificial endothelial cell denudation (Stemerman & Ross. 1976), heat shock (Berberian *et al.* 1990), viral infections (Hajjar *et al.* 1986) and electrical stimulation (Betz & Schlote. 1979). However, none of these models successfully reproduced the development of human atherosclerotic lesions.

Endothelial cell dysfunction describes the imbalance between endothelium-dependent vasodilatation and endothelium-dependent contraction (Mahmoudi *et al.* 2007). Healthy endothelial cells respond to stimuli such as shear stress, thrombin and aggregating platelets by releasing compounds (eg. NO and prostacyclin) which cause relaxation of underlying vascular smooth muscle, and inhibit platelet aggregation

(Lloyd-Jones & Bloch. 1996; Bos *et al.* 2004; Feletou *et al.* 2008). NO also reduces endothelial cell expression of adhesion molecules (thus reducing macrophage adhesion), which prevents vascular smooth muscle cell proliferation and limits the formation of oxidised low-density lipoprotein (oxLDL). When the endothelium is damaged, production and release of NO is reduced, which facilitates the inflammatory response and accelerates formation of atherosclerotic plaques (Lloyd-Jones & Bloch. 1996; Vanhoutte. 2009). Endothelial dysfunction also results in a build up of reactive oxygen and nitrogen species (Ross. 1993; Freeman *et al.* 1995; Harrison. 1997). Excessive production of reactive oxygen species can result in mitochondrial damage in endothelial cells, which then results in whole cell damage as mitochondrial antioxidant enzymes protect healthy cells from damage (Cohen & Kesler. 1999). Mitochondrial damage may provide a link between risk factors, oxidative stress, endothelial dysfunction and the development of atherosclerotic lesions.

1.2.3 Thrombosis

As described, the formation of blood clots at sites of atherosclerotic plaque erosion and rupture is central to the acute life-threatening complications associated with this condition. Therefore, it is important to understand the processes that lead to clot formation.

Prevention of blood loss, while maintaining fluidity of blood within the intact circulation, is essential for maintaining the integrity of the circulatory system. Arrest of blood flow from damaged blood vessels is achieved through a mechanism termed haemostasis. In this process, vessel damage causes vasoconstriction, which reduces blood flow, followed by platelet activation and aggregation. This leads to formation of a haemostatic plug, which is reinforced by fibrin and, in many instances, is sufficient to stop bleeding (Macfarlane. 1940). Thrombosis, by contrast, is the pathological formation of a haemostatic plug within the vasculature, without bleeding or traumatic vessel damage and has been described as “haemostasis in the

wrong place” (Macfarlane. 1977; Gailani & Renne. 2007). Thrombus formation can, in some cases, completely block blood flow to the heart or brain, causing myocardial infarction or stroke. Three major factors (Virchow’s triad), identified over a hundred years ago, influence thrombus formation, size and stability. These are; injury to the vessel wall (generation of a thrombogenic surface), altered blood flow, and abnormal blood coagulability (Fuster *et al.* 2005). Thrombi formed in veins and arteries have very different properties; venous thrombi, which are similar in composition to blood clots that form in static blood *in vitro*, are rich in fibrin and erythrocytes but contain few platelets (see Gailani & Renne. 2007), whereas arterial thrombi consist of platelets and leukocytes in a fibrin mesh, and are usually associated with atherosclerosis (see Gailani & Renne. 2007). Thrombus formation in the vasculature is controlled by activation (and aggregation) of platelets (Gailani & Renne. 2007) and initiation of a cascade of proteolytic reactions leading to the generation of thrombin and formation of a fibrin clot (Macfarlane. 1964; Davie & Ratnoff. 1964). These processes do not occur independently, and there is much ‘cross-talk’ between the two systems to modulate the formation of a stable thrombus (Gailani & Renne. 2007). In healthy individuals, haemostasis is regulated by a complex balance between pro-thrombotic and anti-thrombotic mechanisms (Rosenberg & Aird. 1999). However, coagulant pathways are activated upon rupture of atherosclerotic plaques (as described, Chapter 1.2.3), often leading to the formation of fully occlusive thrombi, which can result in myocardial infarction or stroke. A comprehensive review of the complexities of haemostasis is beyond the scope of this chapter, which will be restricted to consideration of key processes of direct relevance to the experimental investigations. Figures 1.3 and 1.4 outline the key processes of platelet function and the coagulation cascade, while figure 1.5 shows how these processes interact with each other in terms of thrombus formation.

1.2.3.1 Platelet Function

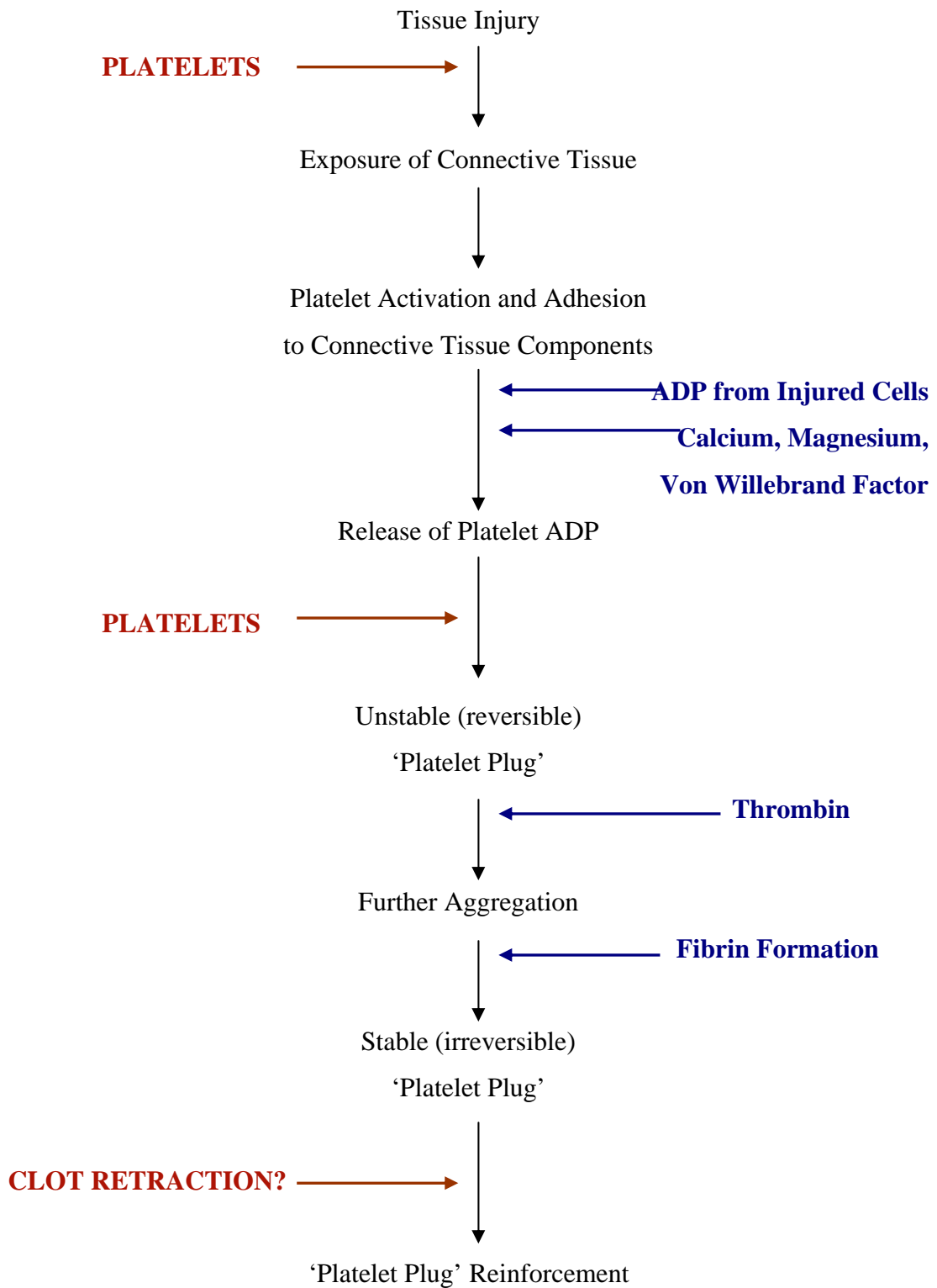
Platelets are small (2-4µm in diameter) disc-shaped cells, containing organelles but no nucleus (Berger. 1970). Platelets are an important factor in haemostasis and

vascular thrombosis, due to their ability to become rapidly activated at sites of vascular injury. A healthy endothelium produces and secretes NO and prostaglandins, which inhibit platelet activation and aggregation (as discussed in Chapter 1.2.1.1. Radomski *et al.* 1987a; Radomski *et al.* 1987b), allowing the continuation of blood flow in undamaged vessels. Upon damage to the vascular wall, platelets interact with subendothelial extracellular matrix, which contains adhesive molecules such as collagen, von Willebrand Factor and integrins (Andre *et al.* 2000; Ruggeri. 2002; Jackson *et al.* 2003; Nieswandt & Watson. 2003; Ruggeri. 2003). Platelet interactions with these molecules cause adhesion to the vessel wall, resulting in the formation of a platelet monolayer (Offermanns. 2006). The recruitment of additional platelets, to form a platelet plug, is mediated via locally-accumulating mediators, such as ADP/ATP and thromboxane A₂, which are released from activated platelets (Offermanns. 2006. Figure 1.3). These mediators act through G protein-coupled receptors on the platelet surface, resulting in a positive feedback loop, which allows platelet coverage of damaged vessels (Offermanns. 2006).

Atherosclerosis is widely considered to be an inflammatory process, with lipids in the vessel wall becoming abnormally oxidised, associated with the local infiltration of leukocytes (Lusis. 2000). In this state, endothelial cell dysfunction, and thus reduced generation of factors inhibiting platelet activation (NO and prostaglandin), contribute to platelet activation that has a role in the initiation of atherosclerosis (see Ruggeri. 2002; Schafer & Bauersachs. 2008). Upon plaque rupture, the highly thrombogenic plaque core is exposed to circulating blood, resulting in a high degree of platelet activation and aggregation, and subsequent activation of the coagulation cascade through exposure of acidic phospholipids (Rosenberg & Aird. 1999. See Figure 1.5), resulting in the generation of thrombin and fibrin (Chapter 1.2.3.2). This is combined with the synthesis and secretion of mediators such as ADP, which creates a positive feedback loop causing further platelet activation (Offermanns. 2006). These processes are activated abnormally in this situation, and cause thrombus formation in the damaged vessel, which can lead to complete vessel occlusion.

Figure 1.3 - Mechanism of Haemostatic Plug Formation.

Mechanism outlining platelet involvement in thrombus formation. Adapted from Berger. 1970.



1.2.3.2 The Coagulation Cascade

Injury to the vessel wall is a major stimulus for coagulation. Injury results in the release of P-selectin and von Willebrand factor to the surface of endothelial cells (McEver *et al.* 1989; Andre *et al.* 2000). P selectin binds to its receptor, P-selectin glycoprotein ligand-1, on the surface of neutrophils and monocytes (Wagner. 2005), which promotes rolling of leukocytes on the surface of activated endothelial cells, concentrating these cells at the area of thrombus formation (Polgar *et al.* 2005). Von Willebrand factor binds to platelet membrane glycoprotein Ib α and to exposed vessel connective tissue, helping to secure adhesion of platelets to the site of injury (Andre *et al.* 2000). The adhesion of platelet-leukocyte aggregates to injured endothelium results in the activation of tissue factor. Tissue factor, along with calcium ions and acidic phospholipids released from activated platelets, is required for the activation of the *extrinsic coagulation pathway* (Rosenberg & Aird. 1999. Figure 1.4). This involves the activation of a series of serine protease enzymes, producing a “cascade” in which each enzyme activates increasing amounts of the next. This process has the ability to convert a small initiating event into a large fibrin clot. The first enzyme to be activated is factor X, to produce factor Xa. Factor Xa, along with factor Va, then converts factor II (prothrombin) into factor IIa (thrombin). Thrombin then converts inactive fibrinogen into fibrin, which stabilises the ‘platelet plug’, and activates factor XIIIa, which stabilises the fibrin mesh. Several other factors are also involved in coagulation, namely factor XIIa, which activates factor XI, which activates factor IX. Factor IXa is also capable of activating factor X through a separate mechanism to tissue factor (Green. 2006). This mechanism is known as the *intrinsic pathway*, it was thought to be activated only when blood comes into contact with an artificial surface such as glass; however recent studies have provided evidence that components of the intrinsic pathway also contribute to regulation of arterial thrombosis (Gailani & Renne. 2007). The extrinsic pathway, also known as the *in vivo* pathway, is directly responsible for controlling blood coagulation and thrombus formation in response to stimuli within the vasculature.

Figure 1.4 - The Coagulation Cascade.

Schematic representation of the coagulation cascade, showing the factors activated in the intrinsic pathway via thrombus formation (tissue activation) and the extrinsic pathway via surface interaction of blood *ex vivo*. Adapted from Pharmacology, 5th Edition. Rang *et al.*

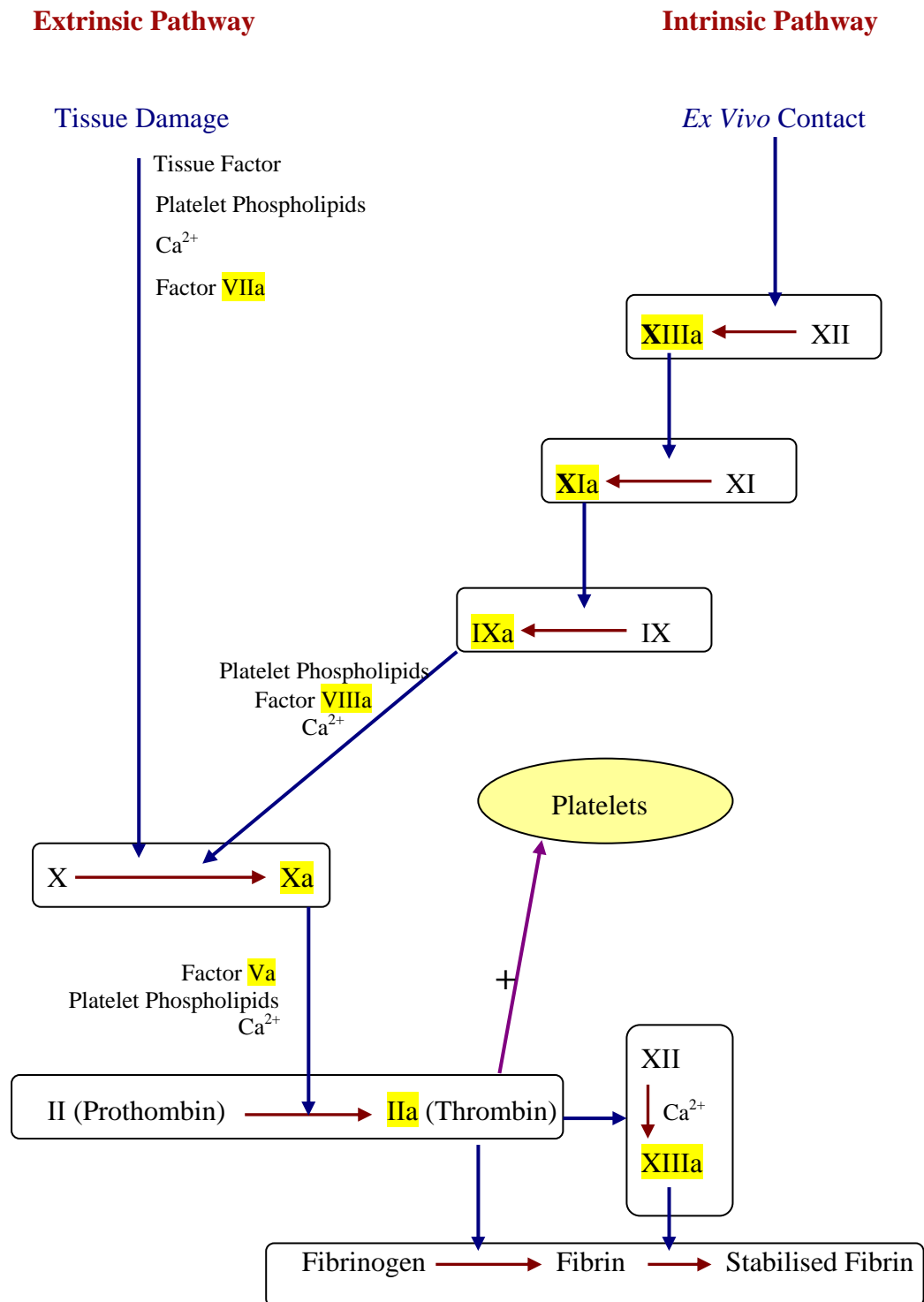
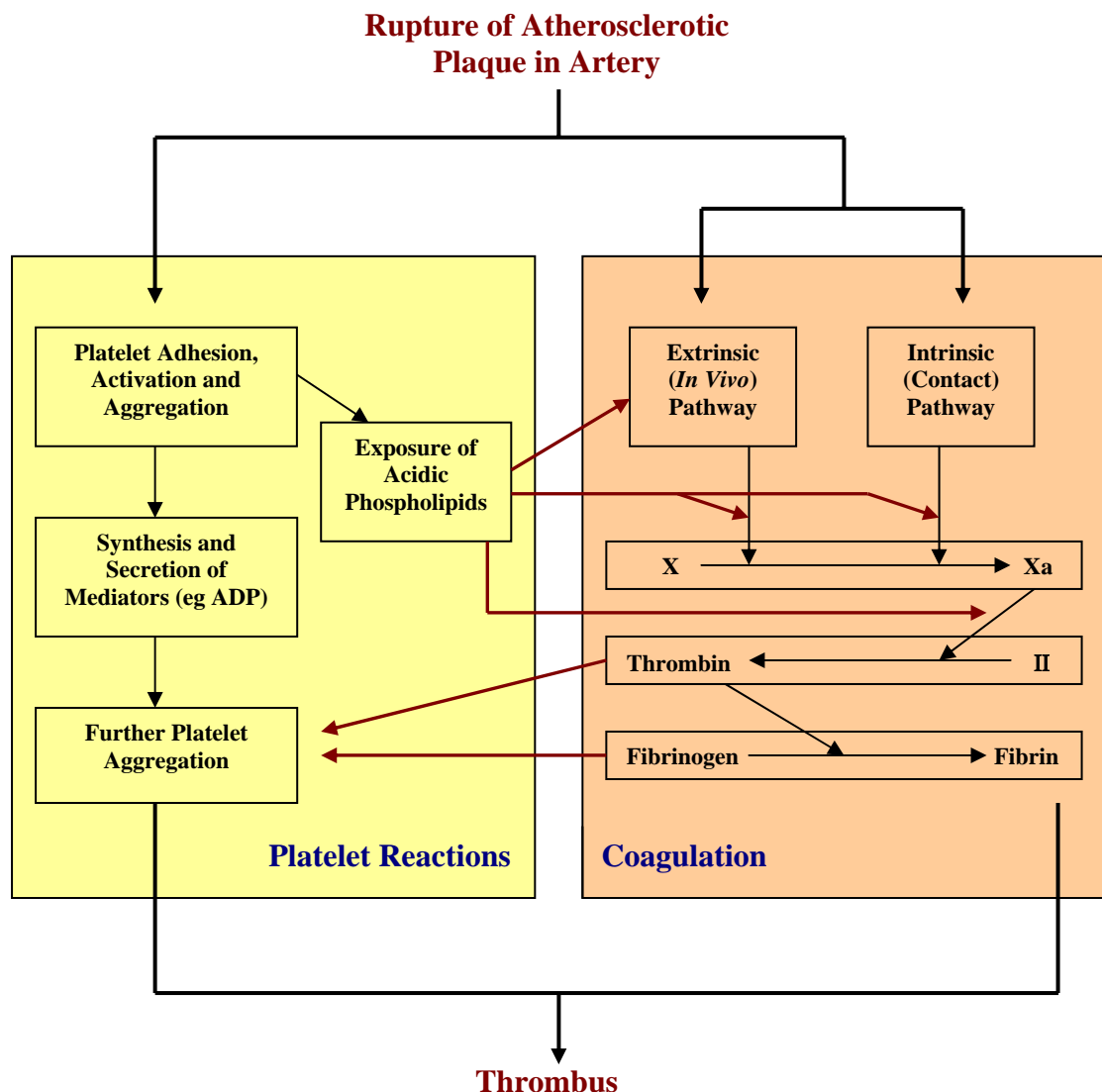


Figure 1.5 - Common Pathways Involved in Activation of Platelets and the Coagulation Cascade.

Interactions between the platelet activation pathway and the coagulation cascade. Red arrows show pathways common to both systems. Acidic phospholipids released from activated platelets cause activation of the extrinsic pathway, increased conversion of factor X to factor Xa (in both the extrinsic and intrinsic pathways) and increased conversion of factor II to thrombin via action on factor Xa. Both thrombin and fibrinogen act on platelets to increase platelet aggregation. Adapted from Pharmacology, 5th Edition. Rang *et al.*



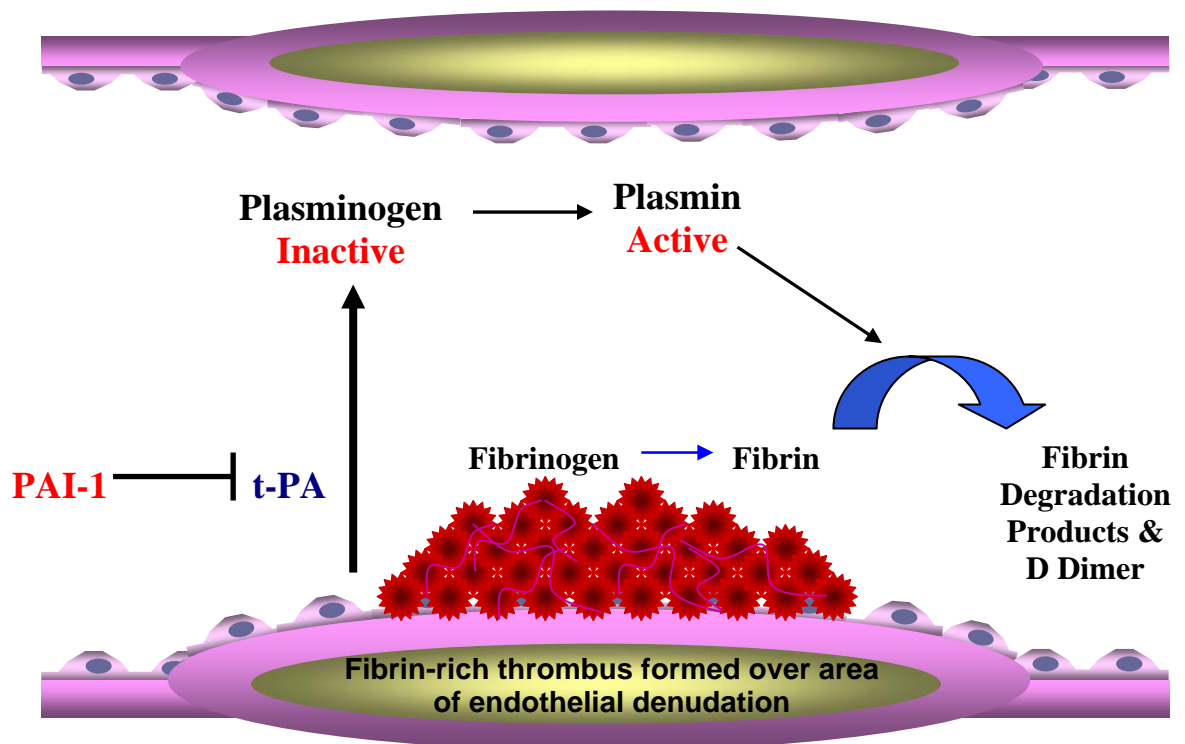
As described, platelet activation and the coagulation cascade occur simultaneously, with many common activation pathways involved. Figure 1.5 shows the crossover points between these two systems.

1.2.3.3 The Fibrinolytic System

The role of the endogenous fibrinolytic system is to prevent abnormal thrombus formation (Figure 1.6). Upon thrombus formation, the pro-enzyme plasminogen is converted to the functional enzyme plasmin, which breaks down fibrin to soluble fibrin degradation products and D Dimer, allowing the thrombus to be dissolved (Lijnen. 2001). Two distinct enzymes have been found which convert plasminogen to plasmin: urokinase plasminogen activator (u-PA) and tissue plasminogen activator (t-PA) (Thorsen & Philips. 1984; Lijnen. 2001). Only t-PA, however, is involved in the dissolution of thrombi in the circulation (Thorsen & Philips. 1984; Lijnen. 2001). t-PA is a single-chain serine proteinase (Pennica *et al.* 1983). The actions of t-PA are regulated by a second enzyme, plasminogen activator inhibitor (PAI-1), which is a single-chain glycoprotein and a member of the serpin family (Kruithof *et al.* 1984; Ny *et al.* 1986; Pannekoek *et al.* 1986). PAI-1 appears to be the major regulator of t-PA action, as naturally occurring complexes form between t-PA and PAI-1, inhibiting the action of t-PA (Rijken *et al.* 1983; Thorsen & Philips. 1984). Most circulating t-PA is inactive and complexed with PAI-1, as circulating PAI-1 levels are significantly greater than t-PA levels; this allows prevention of systemic bleeding while permitting necessary local clot lysis (Voetsch & Loscalzo. 2004). The vascular endothelium plays a significant role in regulation of the fibrinolytic system; endothelial cells synthesise and release fibrinolytic proteins (Levin & Loskutoff. 1982; Cervený *et al.* 1984), and endothelial surface proteins are now thought to modulate the function of fibrinolytic proteins (Hajjar *et al.* 1986; Laiho *et al.* 1986).

Figure 1.6 - Schematic Representation of the Endogenous Fibrinolytic System.

Interactions between factors of the endogenous fibrinolytic system in response to thrombus formation within the vasculature. Tissue plasminogen activator (t-PA) is released from endothelial cells in response to thrombus formation; t-PA converts inactive plasminogen to active plasmin, plasmin in turn causes the breakdown of fibrin (which is involved in thrombus stabilisation) to fibrin degradation products and D dimer, allowing dissolution of the thrombus. t-PA action is inhibited by the production and release of plasminogen activator inhibitor 1 (PAI-1); the balance of t-PA and PAI-1 synthesis and release allows the maintenance of blood flow.



1.2.3.4 Pre-Clinical Investigation of Thrombus formation - Animal Models of Arterial Thrombosis

A number of *in vitro* and *ex vivo* assays are available for investigating blood coagulation and platelet activation. Indeed such assays have been central in clarifying the mechanisms that contribute to thrombus formation and some have important roles in the clinic. However, improving understanding of the processes that regulate thrombus formation *in vivo* has necessitated the development of suitable assays in experimental animals (Day *et al.* 2004; Furie & Furie. 2005). Although animal models have been developed to assess both venous and arterial thrombus formation, it is the latter that is most relevant to the work described in this thesis (as atherothrombosis is an arterial disease). The principle behind most of the models available is simple: induction of a discrete focal injury to the arterial wall causes a thrombotic response that can be assessed in several ways (often by measuring blood flow/arterial occlusion (Day *et al.* 2004; Furie & Furie. 2005)). The main variation tends to be the approach used to cause arterial damage. These techniques have been applied to a variety of animals; use of mice has made it possible to exploit gene targeting of a number of genes encoding proteins involved in development of thrombosis (see Day *et al.* 2004). It should be noted, however, that many (although not all; Xian *et al.* 2009) studies use animals without pre-existing cardiovascular disease, whereas pathological thrombosis in humans is associated with diseased vessels.

Direct application of ferric chloride to the adventitial surface of an artery induces the formation of a platelet-rich thrombus, which typically leads to complete vessel occlusion (Kurz *et al.* 1990). Alternatively, damage to the vascular endothelium can be induced using oxidative stress. This is achieved by intravenous administration of a photo-reactive substance (Rose Bengal) which is then activated by illumination (green light; 540 nm) of an exposed section of a target artery to produce reactive oxygen species (Watson *et al.* 1985). Both these methods induce thrombus formation through endothelial damage and produce reliable, reproducible generation of thrombi. They do not, however, involve arterial stenosis, which is an important factor

in the development of thrombi associated with acute cardiovascular events. In contrast, the Folts model uses both endothelial damage and stenosis to promote arterial thrombus formation and is widely regarded as the pharmaceutical industry ‘gold standard’ (Day *et al.* 2004). It was first developed using dog coronary arteries (Folts *et al.* 1982), and has since been adapted for use in small animals such as mice (Sturgeon *et al.* 2006), rats (Daykin *et al.* 2006; Sturgeon *et al.* 2006) and rabbits (Marret *et al.* 2004; Daykin *et al.* 2006; Sturgeon *et al.* 2006). All small animal models use the carotid, rather than coronary, artery. This is necessary due to the size and accessibility of the coronary artery in small animals, but it also produces more reliable and reproducible results by avoiding the haemostatic disturbance associated with open-chest surgery. The adapted Folts models apply 50% (Daykin *et al.* 2006; Sturgeon *et al.* 2006) or 60% (Marret *et al.* 2004) stenosis to the carotid artery which is then subjected to repeated mechanical injury. This causes de-endothelialisation and exposure of the thrombogenic intimal surface. Blood flow in the carotid artery is monitored until it stops, indicating that an occlusive thrombus has formed at the site of stenosis. The thrombus is then embolised to restore blood flow and allow formation of a new thrombus. This causes repeated changes in blood flow (cyclic flow reductions (CFRs; (Folts 1991)) which are counted over a set period of time to give a quantitative measure of thrombogenicity. The results of the modified Folts model have shown consistency, both within and between experimental groups with CFRs comparable in rats (7.0 ± 0.3 CFRs/ 30 min (Daykin *et al.* 2006; 7.9 ± 0.4 CFRs/ 30 min (Sturgeon *et al.* 2006)), mice (8.7 ± 1.1 CFRs/ 15 min (Sturgeon *et al.* 2006)) and rabbits (4.6 ± 0.2 CFRs/30 min; (Sturgeon *et al.* 2006)). Histological analysis confirms that the thrombi formed using this technique are comparable to human thrombi that form following vessel damage or atherosclerotic plaque rupture.

1.2.4 Atherothrombosis

As described, the fibrinolytic system regulates formation and dissolution of thrombus and, thus, can modulate the thrombotic response to plaque rupture. It seems logical, therefore, that thrombus formation would be more severe following plaque rupture in

individuals with a dysfunctional endogenous fibrinolytic system. This is important, as some cardiovascular risk factors (such as changes in lipid metabolism, cigarette smoking, hyperglycemia, haemostasis) are associated with increased blood thrombogenicity and impaired fibrinolysis (Kullo *et al.* 2000; Edelberg *et al.* 2001; Edgington. 2001; Rauch *et al.* 2001; Salomaa *et al.* 2002). A number of epidemiological studies have also shown a clear association between increased circulating fibrinogen levels and increased incidence of ischemic heart disease (Meade *et al.* 1980; Wilhelmsen *et al.* 1984; Stone *et al.* 1985; Meade *et al.* 1986; Kannel *et al.* 1987a; Kannel *et al.* 1987b; Welin *et al.* 1987; Moller & Kristensen. 1991; Ernst. 1993; Voetsch & Loscalzo. 2004), indicating that increased fibrinogen level is an independent cardiovascular risk factor. Another cardiovascular risk factor linked with altered clotting pathways is dyslipidaemia. For example, high levels of low density lipoproteins, such as are found within atherosclerotic plaques, can increase platelet activity (Aviram & Brook. 1987); patients presenting with primary hypercholesterolemia show increased platelet activation, aggregation and secretion (Brook & Aviram. 1988). In addition, high levels of lipoprotein A (which are associated with increased risk for coronary heart disease) may inhibit fibrinolysis: lipoprotein A is structurally similar to plasminogen and may bind fibrin or inhibit the formation of fibrinolytic proteins (Loscalzo. 1990; Scanu. 1991).

One of the clearest associations between a cardiovascular risk factor and the fibrinolytic system is provided by cigarette smokers (a group of particular relevance when considering the effect of inhaled pollutants). Smokers have higher levels of fibrinogen (Miller. 1992) and lower levels of t-PA (Newby *et al.* 1999; Newby *et al.* 2001) than non-smokers, plus a considerably increased risk of myocardial infarction. Interestingly, due to their low circulating t-PA levels, smokers are more likely to respond to thrombolytic treatment with t-PA post-myocardial infarction (Newby *et al.* 1999; Newby *et al.* 2001). Whether an equivalent of this “smoker’s paradox” exists in individuals exposed to high levels of air pollution for extended periods has not been shown. Thus atherothrombosis, which is one of the most significant causes of death and disability in the developed (and increasingly in the developing) world, is exacerbated by factors that promote coagulation. This is consistent with the

suggestion that the increased incidence of cardiovascular events in individuals following acute exposure to particulate atmospheric pollution is caused by an increased propensity to thrombosis at sites of atherosclerotic plaque erosion/rupture.

1.3 Nanoparticulates and Thrombosis

Thus far it has been shown that exposure to atmospheric pollution, in particular to combustion-derived fine particulate matter, can increase cardiovascular morbidity and mortality. This can be attributed to the chronic effects of exposure, which accelerate atherogenesis, and/or short-term effects, which cause acute cardiovascular events (including myocardial infarction). The role of thrombosis in the development of myocardial infarction and stroke, combined with increasing evidence that airborne combustion-derived particulate matter increases blood coagulability, suggests that enhanced thrombosis may contribute to the effects of acute exposure. However, despite an increasing number of investigations over the past 10 years, the nature of the interaction between pollution particulate and the thrombotic system has not been established. In order to investigate this relationship, a number of practical issues need to be addressed.

1.3.1 Choice of Particulate for Investigating the Effects of Combustion-Derived Pollutants on Thrombosis

Many different nanoparticles have been investigated for their effects on thrombosis. As discussed earlier (Chapter 1.1.2.1) particles occurring in air pollution include diesel exhaust particulates (DEP) and concentrated ambient particles (CAPs). Both have been studied for their effects on thrombosis (Vincent *et al.* 2001; Nemmar *et al.* 2002c; Nemmar *et al.* 2003b; Nemmar *et al.* 2003c; Mills *et al.* 2005; Carlsten *et al.* 2007; Mills *et al.* 2007; Lucking *et al.* 2008; Mills *et al.* 2008) but these investigations are limited, as the mechanism responsible for the effects of DEP have

still to be fully established. In addition to these pollution-derived particles, many studies have also used nanocarbon black (CB), often as a control particle (Vincent *et al.* 2001; Donaldson *et al.* 2005; Yamawaki *et al.* 2006). CB is a clean carbon nanoparticle, which, although not occurring naturally, can be used to distinguish effects of the carbon core from those of the metals and organic compounds associated with the surface of DEP. An alternative approach has been to use nanoparticles that have been artificially charged (either positively using carboxylates or negatively using amines) in order to investigate the effects of charged particles on the vascular system (Nemmar *et al.* 2002a; Nemmar *et al.* 2003b), due to the fact that a large proportion of ambient particles are charged (Cohen *et al.* 1998), and that the acidic nature appears to play an important role in particle toxicity (Chen *et al.* 1992; Dockery *et al.* 1992). Finally, medical nanoparticles, such as quantum dots, have also been investigated for their potential to promote thrombus formation (Geys *et al.* 2008). Quantum dots are nanoparticle crystal semiconductors that emit visible light upon excitation. They can be bio-engineered to specifically target molecules in order to track generation or breakdown *in vivo* and, consequently, have many exciting potential medical applications. Although quantum dots are not yet being used to study disease in humans, it is important to ensure that they do not have effects on thrombus formation similar to those reported with other nanoparticles.

1.3.2 Particle Administration

Many approaches have been used for exposing both humans and animals to various particles. In clinical trials the most appropriate method of exposure is inhalation, as it most accurately recreates the effects of real life particulate exposure. In many of the acute clinical trials described below (Chapter 1.3.4.1), exposure was achieved using a specially designed diesel exposure chamber (Mills *et al.* 2005; Mills *et al.* 2006; Mills *et al.* 2007; Lucking *et al.* 2008; Mills *et al.* 2008; Tornqvist *et al.* 2008). In contrast, exposure by inhalation is often difficult in pre-clinical studies, as animals pose problems not encountered when using human volunteers. Rodents, for example, have a more complex nasal passage than humans, meaning that nasal hairs and cilia

filter out a considerable quantity of particulate pollution before entering the lungs (Morgan & Monticello. 1990). Also, if whole body exposures are being used, a substantial proportion of the particulate matter may become trapped in the animals' fur, thus reducing the amount inhaled (Morgan & Monticello. 1990). Consequently, and in contrast to controlled human exposures, the amount of particulate reaching the lungs cannot be easily calculated. Intratracheal instillation was developed to avoid these problems and thereby allow delivery of a known quantity of particulate to the lungs. Using this method, the animal is temporarily anaesthetised and the trachea briefly cannulated to allow administration of a suspension of the relevant particulate (Nemmar *et al.* 2002a; Nemmar *et al.* 2003a; Nemmar *et al.* 2003b; Nemmar *et al.* 2003c; Nemmar *et al.* 2004a; Nemmar *et al.* 2004b; Nemmar *et al.* 2005; Geys *et al.* 2008). This method has proved to be very reliable, ensuring minimum discomfort to the animals, which are allowed to recover from the anaesthetic before the start of experiments. Another technique used with experimental animals is the direct injection of particle suspensions into the bloodstream (Nemmar *et al.* 2002a; Nemmar *et al.* 2009). This approach allows examination of the direct effects of particles on the cardiovascular system. This can be used, therefore, to model the potential action of particles that translocate from the lungs into the bloodstream (if this occurs; see Chapter 1.3.5.3). Using both mechanisms of animal exposure it should be possible to distinguish those effects mediated by pulmonary exposure to particulate matter from those caused by direct effects of particles that may translocate into the bloodstream.

1.3.3 Chronic Particulate Exposure

Few chronic exposure studies investigating the cardiovascular effects of particles are available in the literature. Clinical studies appear to be limited to the epidemiological (cohort) approaches described earlier (Chapter 1.1.3). Indeed, given the evidence available from these, chronic human exposure to particulate matter would be ethically unacceptable. Pre-clinical studies in this area are also limited. Stinn *et al.* (2005) exposed rats to tobacco smoke and diesel engine exhaust via inhalation for 6

hours a day, 7 days a week, for 2 years. After this extended period it was shown that exposure to diesel engine exhaust caused pulmonary inflammation, which was not unexpected. However, no investigations were made into thrombogenicity in these animals. Sun *et al.* (2005) showed that a 6 month regimen of PM_{2.5} exposure using apolipoprotein E^{-/-} mice accelerated the progression of atherosclerosis, as well as altering vasomotor tone and inducing vascular inflammation. A similar study conducted in C57BL/6 mice (Sun *et al.* 2009) showed that PM_{2.5} inhalation increases systemic inflammation. However, no chronic pre-clinical studies have fully investigated the long term effects of particulate matter on thrombus formation or endogenous fibrinolysis.

1.3.4 Acute Particulate Exposure

Acute exposures of subjects to pollutant particles are obviously more straightforward and cheaper (and, for humans, more ethically acceptable) than chronic exposures. It is unsurprising, therefore, that a greater number of investigations (both clinical and pre-clinical) into the cardiovascular impact of acute exposures to particulates are available in the published literature.

1.3.4.1 Clinical Studies

Following on from epidemiological studies, clinical trials have become a useful tool for clarifying the mechanisms by which human subjects react to DEP exposure at environmental levels. A number of clinical studies have investigated the effects of inhaled particles both on healthy human volunteers and on those with stable coronary disease (outlined in Table 1.2). Many of these studies have been conducted by the clinical research department at the University of Edinburgh and associated institutions. The first in this series of studies was conducted by Mills *et al.* (2005). In this study, 30 healthy males were exposed to inhalation of DEP (300mg/m³) and filtered air in a randomised double-blind trial for 1 hour with intermittent exercise.

This study showed that endothelium-dependent (bradykinin, acetylcholine) and -independent (sodium nitroprusside and verapamil) vasodilatation were impaired in the forearm 2 and 6 hours after exposure. These results were reinforced by a subsequent investigation, using 20 healthy male volunteers, which demonstrated impaired endothelial function in the forearm 24 hours after inhalation of DEP (300mg/m³ for 1 hour; Tornquist *et al.*, 2007). Mills *et al.* (2005) also showed that infusion of bradykinin into the forearm caused a dose-dependent increase in plasma t-PA, which was suppressed 6 hours after exposure to DEP, indicating dysregulation of the fibrinolytic system; these results were remarkably similar to those observed by Newby *et al.* (1999) when studying the effects of cigarette smoking on the endogenous fibrinolytic system, indicating that similar mechanisms may be at work during both exposures. The ability of DEP to influence coagulation pathways was also demonstrated in a study that exposed 20 healthy human volunteers to inhalation of DEP (350mg/m³) or filtered air for 1 or 2 hours, with moderate exercise, in a randomised double-blind trial (Lucking *et al.*, 2008). DEP was shown to increase thrombus formation *ex vivo* (in the Badimon chamber), apparently as a result of increased platelet-neutrophil and platelet-monocyte aggregation (measured using flow cytometry). Since the acute cardiovascular effects of pollution-derived particulate matter are more likely to be evident in patients with pre-existing cardiovascular disease, some studies have been performed using volunteers from this patient population. Mills *et al.* (2007) examined the responses of 20 males with prior myocardial infarction when exposed to inhalation of DEP (300mg/m³) or filtered air for 1 hour, with intermittent exercise, in a randomised double-blind trial. DEP promoted myocardial ischemia and inhibited the endogenous fibrinolytic system by reducing acute release of t-PA from endothelial cells. A later study showed that inhaled concentrated ambient particles (190±37mg/m³; 2 hours) did not alter vasomotor or fibrinolytic function in 12 healthy males or in 12 male patients with stable coronary heart disease (Mills *et al.*, 2008). It should be noted, however, that the concentrated ambient particles (collected from Edinburgh) were shown to comprise mainly sea salt, which would not be expected to influence cardiovascular function.

Table 1.2 - Overview of Clinical Studies Investigating the Acute Effects of Particulate Exposure.

Subjects	Route of Administration	Timing	Particle	Dose	Effects	Reference
30 healthy males	Inhalation	1 hour with intermittent exercise	DEP and filtered air	300mg/m ³	DEP impaired regulation of vascular tone and endogenous fibrinolysis	Mills <i>et al.</i> 2005
20 healthy volunteers	Inhalation	1 and 2 hours with moderate exercise	DEP and filtered air	350mg/m ³	DEP increased <i>ex vivo</i> thrombus formation and caused <i>in vivo</i> platelet activation	Lucking <i>et al.</i> 2008
15 healthy males	Inhalation	1 hour exposure, studies continue for 24 hours	DEP and filtered air	300mg/m ³	DEP impaired endothelium-dependent vasodilatation and mild systemic inflammation 24 hours after exposure	Tornqvist <i>et al.</i> 2007
20 males with prior myocardial infarction	Inhalation	1 hour with intermittent exercise	DEP and filtered air	300mg/m ³	DEP promoted myocardial ischemia and inhibited endogenous fibrinolysis	Mills <i>et al.</i> 2007
12 healthy males; 12 males with stable coronary heart disease	Inhalation	2 hours	Concentrated ambient fine and ultrafine particles and filtered air	190±37mg/m ³	Concentrated ambient particles did not affect vasomotor or fibrinolytic function in healthy volunteers or patients with coronary heart disease	Mills <i>et al.</i> 2008

These studies support the hypothesis that exposure to DEP, at levels that could be found in cities and built-up areas, causes changes in the cardiovascular system. These include alterations of the endogenous fibrinolytic system and increased platelet activation as well as direct evidence of increased thrombus formation. This is consistent with the contention that exposure to particulate pollution increases myocardial infarction by increasing the risk of acute thrombosis.

1.3.4.2 Pre-Clinical Studies

Several pre-clinical studies have investigated the acute effects of particle exposure on thrombus formation (summarised in Table 1.3). A number of these studies were performed by Nemmar and colleagues, who assessed the effects of intratracheal DEP instillation on acute thrombus formation. In 2003, they reported that instillation of DEP (5-500µg/animal) caused pulmonary inflammation and rapid platelet activation (measured by platelet aggregometry) in hamsters 30 minutes and 1 hour after exposure (Nemmar *et al.* 20003b). In a later study, a similar exposure regiment increased photochemical (Rose Bengal) thrombus formation *in vivo* 1 hour after exposure. As in the earlier study, increased thrombus formation appeared to be caused by platelet activation. Further investigations have suggested that increased thrombus formation in response to DEP instillation (50µg/animal) remained evident up to 24 hours after exposure (Nemmar *et al.* 2003c; Nemmar *et al.* 2004b) and was associated with pulmonary inflammation and histamine release from mast cells. Initially, these studies indicated that pulmonary inflammation and thrombus formation may be partially linked. However, whilst the earlier study (Nemmar *et al.* 2003c) showed that both fine and ultrafine particles caused pulmonary inflammation, only ultrafine particles increased thrombus formation. Finally, administration of DEP (0.01 and 0.02mg/kg; 24 hours) by tail vein injection (to bypass the lungs) caused cardiac changes, which appeared to be associated with systemic and pulmonary inflammation, even though DEP was never in direct contact with the lungs (Nemmar *et al.* 2009).

Table 1.3 - Overview of Pre-Clinical Studies Investigating the Acute Effects of Particulate Exposure.

Animal	Model	Route of Administration	Timing	Particle	Dose	Effects	Reference
Hamster	Platelet aggregometry	Intratracheal instillation	30 min , 1 hr	DEP	5-500µg/animal	Lung inflammation and rapid platelet activation	Nemmar <i>et al.</i> 2003b
Hamster	Rose Bengal	Intratracheal instillation	1, 6 and 24 hours	DEP	50µg/animal	Increased thrombosis not associated with pulmonary inflammation	Nemmar <i>et al.</i> 2003c
Hamster	Rose Bengal	Intratracheal instillation	1 hour	DEP	5-500µg/animal	Enhanced platelet activation and thrombus formation.	Nemmar <i>et al.</i> 2004a
Hamster	Rose Bengal	Intratracheal instillation	24 hours	DEP	50µg/animal	Thrombosis caused by DEP can be prevented by inhibiting histamine release from mast cells	Nemmar <i>et al.</i> 2004b
Spontaneously hypertensive rats	Blood pressure and heart rate	Tail vein injection	24 hours	DEP	0.01, 0.02mg/kg	Cardiac and systemic changes with pulmonary inflammation	Nemmar <i>et al.</i> 2009
Hamster	Rose Bengal and platelet aggregometry	Intravenous injection and intratracheal instillation	10 min	Negatively charged carboxylate modified, positively charged amine modified and unmodified polystyrene UFPs	5-500mg/kg	Platelet activation resulting in increased thrombogenicity with positively charged particles	Nemmar <i>et al.</i> 2002a
Hamster	Rose Bengal	Intratracheal instillation	1 hour	Negatively charged carboxylate modified, positively charged amine modified and unmodified polystyrene UFPs	5-500µg/animal	Positive UFPs cause pulmonary inflammation and enhance thrombus formation	Nemmar <i>et al.</i> 2003a
Hamster	Rose Bengal	Intratracheal instillation	24 hours	Silica particles	2-200µg/animal	Platelet activation via systemic release of neutrophil elastase	Nemmar <i>et al.</i> 2005
Rat	Ferric Chloride, platelet aggregometry and flow cytometry	Not Stated in Paper	Not Stated in Paper	Multiplewall and singlewall carbon nanoparticles, carbon nanoparticles	Not Stated in Paper	Carbon nanoparticles activate platelets and enhance vascular thrombosis	Radomski <i>et al.</i> 2005
Mouse	Platelet aggregometry	Intravenous injection	1, 4 and 24 hours	Amine and carboxylated Quantum Dots	1.44-3,600pmol/animal	Pulmonary vascular thrombosis possibly via activation of the coagulation cascade	Geys <i>et al.</i> 2008

In addition to these experiments using DEP, Nemmar and colleagues have also investigated the effects of charged particles on platelet activation and thrombus formation, using negatively-charged carboxylate-modified, positively-charged amine-modified and unmodified polystyrene ultrafine particles (Nemmar *et al.* 2002a; Nemmar *et al.* 2003a). These studies showed that, while instillation of uncharged and negatively-charged particles had no effect, positively-charged particles caused platelet activation, pulmonary inflammation and increased thrombogenicity. Ultrafine silica particles were also found to cause platelet activation 24 hours after instillation, which was thought to be caused by systemic release of neutrophil elastase (Nemmar *et al.* 2005). Other studies have also found that carbon nanoparticles (Radomski *et al.* 2005) and quantum dots (Geys *et al.* 2008) have the ability to enhance vascular thrombosis via activation of platelets and the coagulation cascade. These studies indicate, therefore, that several different types of nanoparticulate have the ability to increase thrombus formation although the mechanisms involved remain unclear.

1.3.5 How Does Particulate Air Pollution Alter Cardiovascular Function?

As described, there is increasing evidence that exposure to particulate matter can alter the function of the cardiovascular system and, in particular, can increase thrombus formation by the blood. However, whilst it appears that air pollution causes an increase in plasma viscosity (Peters *et al.* 1997), perhaps due to increased plasma concentrations of fibrinogen (Ghio *et al.* 2000, 2003; Ulrich *et al.* 2002), the precise mechanisms involved remain the subject of debate. Some effects of exposure to particulates, such as the PM-induced changes in heart rate (Pope *et al.* 1999b), indicate that pollution induces dysregulation of the autonomic nervous system (Simkhovich *et al.* 2008). In a recent review, however, Mills & colleagues distilled current understanding into two main pathways that might underpin particulate-mediated changes in vascular function: the “classical” pathway in which cardiovascular abnormalities are induced via inflammatory mediators, and an “alternative” pathway, in which particulates translocate into the circulation and interact directly with components of the cardiovascular system (Mills *et al.* 2009).

1.3.5.1 Particulate-Induced Inflammation

Pulmonary inflammation is a well-known effect of pulmonary exposure to ultrafine particles, including DEP. Inhalation of CAPs in human subjects causes mild inflammation in the lower respiratory tract (Ghio *et al.* 2000). Similarly, intratracheal instillation of DEP increases pulmonary expression of a number of inflammatory factors, including cytokine-induced neutrophil chemoattractant-1 (CINC-1), tumour necrosis factor- α (TNF α), macrophage inflammatory protein-2 (MIP-2. Singh *et al.* 2004; Yokota *et al.* 2005; Yokota *et al.* 2008), cyclooxygenase-2 (COX-2) and prostaglandin E₂ (Cao *et al.* 2007; Ahn *et al.* 2008). Significant increases in pulmonary neutrophils, lymphocytes and total lavage protein have also been observed following particulate instillation (Clarke *et al.* 1999; Yokota *et al.* 2008). It has been proposed that pulmonary inflammation due to DEP is mediated through the production of peroxynitrite, initiated via DEP-mediated up-regulation of inducible nitric oxide synthase (Ito *et al.* 2000). Instilled silica particles also produce pulmonary macrophage-neutrophil cross talk, causing the release of neutrophil elastase into the bloodstream. Elastase acts to trigger the activation of circulating platelets, potentially providing a link between pulmonary inflammation and the initiation of thrombotic events (Nemmar *et al.* 2005).

Systemically administered particles, including DEP, have been shown to cause systemic inflammation with increased expression and activity of inflammatory factors such as superoxide dismutase, IL-6 (Nemmar *et al.* 2009; Nemmar *et al.* 2010), tumour necrosis factor (TNF α) and leukotriene B₄ (Nemmar *et al.* 2009). Systemic administration of particulates also increases the number of circulating monocytes and granulocytes (Nemmar *et al.* 2008). However, these direct affects on systemic inflammation may not apply when subjects are exposed to DEP via inhalation or instillation. Seaton *et al.* (1995) suggested that deposition of ultrafine particles in the alveoli causes local inflammation leading to a systemic inflammatory response, which can exacerbate cardiovascular conditions in susceptible individuals (Seaton *et al.* 2005). In this model, inhalation of ultrafine particles causes an increase in inflammatory cells in the lungs, which release proinflammatory cytokines and chemokines, including IL-8, TGF- β 1 and C-reactive protein, into the circulation.

These then trigger a cascade of inflammatory reactions throughout the body leading to accelerated atherogenesis and altered cardiac function (Salvi, *et al.* 1999; Abe *et al.* 2000; Donaldson *et al.* 2001; van Eeden *et al.* 2001; Fujii *et al.* 2002; Goto *et al.* 2004; Pope *et al.* 2004). Exposure to ultrafine particles has also been shown to increase reactive oxygen species, promoting an oxidative stress response, which may promote the progression of atherosclerosis and increase the severity of acute cardiovascular responses such as hypertension and myocardial infarction (Delfino *et al.* 2005). Instilled DEP has also been shown promote systemic inflammation via increased levels of CINC-1, causing an increase in systemic neutrophils and oxyradical production (Yokota *et al.* 2005). These studies suggest that systemic inflammation may be a secondary reaction to pulmonary inflammation caused by particulate exposure, and may provide an insight into the mechanisms responsible for the increased incidence of cardiovascular events following human exposure to airborne particulate pollution.

1.3.5.2 Translocation of Ultrafine Particles

The second theory is that UFPs are able to translocate from the alveoli into the blood to affect the heart and vasculature directly. Extrapulmonary translocation of radiolabelled ultrafine polystyrene particles has been shown in rats by measuring the amount of radioactivity collected in the blood and organs following instillation (Chen *et al.* 2006). This study also showed that pre-treatment with lipopolysaccharide induced pulmonary inflammation and markedly increased the rate of translocation, suggesting that inhaled UFPs reach the alveoli and cause inflammation, which makes the alveoli more permeable and allows particles to enter the bloodstream (Chen *et al.* 2006). Similar results have also been shown in healthy human volunteers; radiolabelled ultrafine carbon particles (Technegas) could be detected in the blood of the volunteers 1 minute after inhalation (Nemmar *et al.* 2002). However, a study by our group suggested that extrapulmonary translocation of this particular particle does not occur, and instead the radiolabel leached off the ultrafine particles before entering the bloodstream (Mills *et al.* 2006). Whether ultrafine diesel particles can pass into the blood still remains unclear.

1.4 Summary

This introduction outlines the potential link between exposure to air pollution, in particular nanoparticulate pollution, and increased morbidity and mortality due to cardiovascular incidents. Many epidemiological studies have shown that exposure to airborne pollution (a significant component of which comes from combustion of fossil fuels) results in an increased risk of myocardial infarction. However until recently the mechanism at work could only be speculated. Practical constraints have ensured that there are few clinical or pre-clinical studies into the cardiovascular effects of chronic exposure to particulate pollutants. Considerably more investigations have addressed the cardiovascular consequences of acute exposure to particulates. These suggest that DEP can increase thrombus formation by causing platelet activation and inhibiting the endogenous fibrinolytic system. Whether pulmonary and systemic inflammation are necessary mediators of these changes remains unclear, as does the role of particle translocation from the lungs to the systemic circulation. Particle-induced increase in thrombus formation could predispose at-risk individuals to thrombosis at sites of atherosclerotic plaque rupture. This would explain the increased cardiovascular events observed in patients following acute exposure to atmospheric pollution. The work described in this thesis was designed to clarify the mechanisms through which exposure to DEP increases the likelihood of thrombus formation.

1.5 Hypothesis and Aims

The work described in this thesis addressed the hypothesis:

Exposure to diesel exhaust particulate increases thrombosis, by activating platelets and inhibiting endogenous fibrinolysis, independent of pulmonary and systemic inflammation.

The specific aims were to:

- Develop a suitable *in vivo* model for assessing the effects of DEP exposure on thrombus formation.
- Use this model to investigate the effects of DEP (and suitable control particles) on thrombus formation and pulmonary and systemic inflammation.
- Clarify the importance of platelet activation and inhibition of the endogenous fibrinolytic system to the DEP-mediated increase in thrombus formation.
- Determine whether exposure to DEP inhibits the activity of the endogenous fibrinolytic system in cultured endothelial cells.

Chapter 2

Materials & Methods

2.1 *In Vivo* Models of Thrombus Formation

2.1.1 Animals

Male Wistar rats (150-200g) were obtained from Charles River Laboratories (Wilmington, MA, USA). All animals were housed for at least one week, with constant access to food and water and a 12 hour light/dark cycle, before use in the following experiments. All animal experiments were licensed and followed the Home Office Procedures Act.

2.1.2 Preparation and Administration of Particulate Suspensions

2.1.2.1 Preparation of Particulate Suspensions

Diesel exhaust particulates (DEP) collected from industrial forklift engine exhausts (SRM 2975) were obtained from the National Institute of Standards and Technology (NIST; Gaithersburg, MD, USA). Carbon black particles (CB; Degussa, Germany) and quartz particles (DQ12; IUF, Germany; Robock. 1973) had previously been baked at 290°C for 8 hours to ensure the particles were free of contaminants, especially lipopolysaccharide (LPS). DEP could not be baked, as this would remove volatile chemical compounds from the particle surface. Instead DEP was regularly tested for LPS via Limulus Amebocyte Lysate assay (QCL-1000 Endotoxin Assay; Cambrex, NJ, USA) according to the manufacturers instructions. Dr Catherine Shaw also recently tested CB and DEP particles, and were both found to be completely LPS free. Solutions of DEP (1mg/ml), CB (1mg/ml) and DQ12 (0.25mg/ml) were prepared in sterile 0.9% saline by sonication (Status Homogenisers. Probe from Philip Harris Scientific, US70; Lichfield, UK) at 70% power for 5 minutes. All particle suspensions were prepared immediately prior to use. The comparison of CB with DEP would allow observations of the effects of the carbon core versus the associated compounds on the DEP surface, as CB is a clean carbon particle. DQ12 is a large particle that induces pulmonary inflammation; its concentration in the following experiments was chosen to induce a similar level of pulmonary inflammation as the corresponding DEP and CB doses, based on previous work (Dr.

Rodger Duffin, Centre for Inflammation Research, University of Edinburgh; Duffin *et al.* 2001).

2.1.2.2 Pulmonary Instillation

Animals were anaesthetised using isoflurane (Merial; Essex, UK) and suspensions (0.5ml) containing the relevant amount of particulate (DEP, CB, DQ12) or vehicle (0.9% sterile saline) were applied directly into the lungs by intratracheal instillation (Duffin *et al.* 2001). Briefly, animals were temporarily anaesthetised by isoflurane inhalation, cannulated with a paediatric laryngoscope to expose the trachea, and 0.5ml of saline or suspensions containing the experimental particles were instilled into the lungs before the animal regained consciousness. Animals were allowed to recover from the anaesthetic and maintained for 2, 6 or 24 hours under normal housing conditions before being assessed for thrombus formation and markers of inflammation (see below, Chapter 2.1.3). Instillations were performed by Dr Rodger Duffin (Centre for Inflammation Research, University of Edinburgh).

2.1.2.3 Intravenous Administration

Intravenous administration of experimental solutions and saline was carried out by tail vein injections. Animals were weighed and received a bolus (0.5ml) of DEP or CB suspensions (0.5mg/kg) while conscious and restrained. Control animals received a comparable volume of vehicle (saline). Animals were allowed to recover from the procedure for 2, 6 or 24 hours before being anaesthetised and assessed for thrombus formation and markers of inflammation (see below; Chapter 2.1.3). Injections were performed by Keith Chalmers (BRR-BRF, The University of Edinburgh).

2.1.3 Models of Thrombosis

2.1.3.1 Ferric Chloride-Mediated Induction of Thrombosis

Induction of arterial thrombus by direct application of ferric chloride was developed in male Wistar rats (Body weight 175g and 275g) by adaptation of the method described by Kurz *et al.* (1990). Rats were anaesthetised and maintained via inhalation of isoflurane, and prepared for surgery by shaving the anterior surface of the neck. The left carotid artery was exposed by blunt dissection and carotid artery flow measured by emplacement of an ultrasonic flow probe (Transonic Systems Inc; Maastricht, Netherlands). Ferric chloride was applied topically to the carotid artery; this was achieved using a cylinder of plastic tubing (2mm internal diameter) approximately 3mm in length. The tubing was cut open and a strip of Whatman filter paper (No 5) was placed inside and folded to create a double layer. The tubing and filter paper were then dipped in 20% ferric chloride solution (BDH; Poole, UK) and placed around the carotid artery. Care was taken to ensure full contact between the filter paper and the artery wall. The ferric chloride solution was left in contact with the artery for 10 minutes before being carefully removed. Blood flow through the artery was recorded, timing was started when ferric chloride was applied to the artery and was stopped when blood flow reached 0ml/min.

2.1.3.2 Time to Arrest of Bleeding

In all animals, the time to arrest of tail tip bleeding (Tanaka *et al.* 1998) was assessed once total occlusion of the carotid artery had been achieved. The tail tip (~5mm) was removed using a scalpel and cleaned every 10 seconds using a swab (Medical Wire and Equipment Company; Wiltshire, UK). Arrest of bleeding was considered to be the time when no blood was transferred from the tail to the swab.

2.2 Histological Analysis of Thrombus Formation and Particle Translocation

Following surgery, aorta, carotid artery, kidney, liver and lung sections were removed and fixed in neutral buffered formalin (24 hours) before being processed and embedded in paraffin wax for histological analysis. Serial sections (4µm) were cut using a microtome and the sections were stained using the haematoxylin and eosin method to look at organ/vessel structure and thrombus (Lillie *et al.* 1976). Sections mounted on glass microscope slides (Superfrost Plus, 75mm x 25mm. VWR International Ltd; Leicestershire, UK) were rehydrated through a series of alcohol steps at reducing concentrations, then rinsed in distilled water. Nuclei were then stained using haematoxylin and the samples were differentiated using 0.3% acid alcohol. Sections were again rinsed in distilled water, then stained with eosin and rinsed again. Following staining, sections were dehydrated through a series of alcohols of increasing concentration. Samples were then cover-slipped using DPX solution (Sigma Aldrich; Dorset, UK) and photographed at 100x and 400x magnification, and compared with lung sections, taken 2 hours after DEP instillation, for identification of DEP.

2.3 The Impact of Particulate Administration on Pulmonary Inflammation

Intratracheal administration of particulate solutions was expected to induce pulmonary inflammation, whereas intravenous administration was not. A number of complementary methods were used to assess the nature of pulmonary inflammation in response to exposure to different particulate solutions.

2.3.1 Collection and Processing of Plasma and Bronchoalveolar Lavage Fluid

Anaesthetised rats, exposed to particulate solutions or vehicle, were killed by exsanguination; blood was removed into a syringe, via the abdominal aorta, and mixed with 3.8% sodium citrate solution (dilution factor 10:1). Whole blood was centrifuged (1500g, 5 minutes, room temperature) to obtain plasma, which was separated into aliquots (0.5ml) and stored at -80°C until further analysis. The lungs were then cannulated and lavaged with (1 × 8ml) sterile saline. This first lavage was retained in a 15ml falcon tube for cellular analysis of the bronchoalveolar lavage (BAL) fluid profile. The lungs were then lavaged a further 3 times with sterile saline (8ml) and the BAL fluid from these lavages was combined in a 50ml falcon tube (Duffin *et al.* 2001; Duffin *et al.* 2007). All samples were centrifuged (180g, 5 minutes, 4°C). The supernatant from the first lavage was separated into 1ml aliquots and stored at -80°C for further analysis. The supernatant from the 50ml falcon tube was removed and discarded, and the cell pellet was resuspended in 1ml sterile saline and added to the cell pellet from the first lavage.

2.3.2 Cell content of Bronchoalveolar Lavage fluid

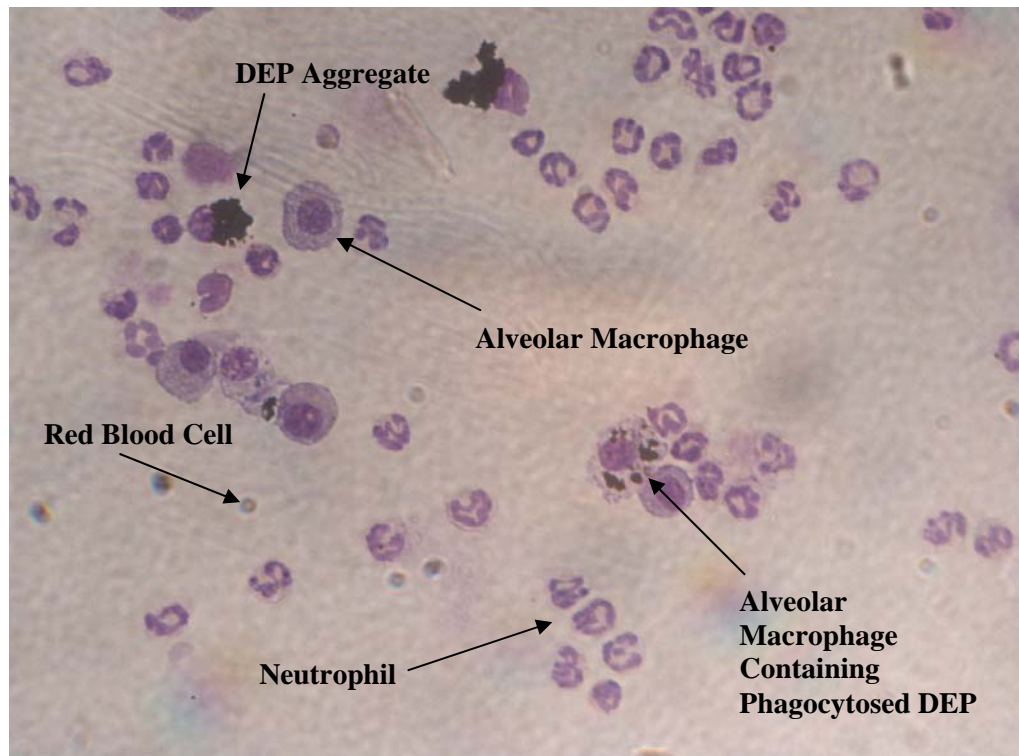
Cell pellets obtained from BAL fluid (see above; Chapter 2.3.1) were re-suspended (in 1ml sterile saline) and analysed using an automatic cell counter using standard settings (ChemoMetec A/S; Denmark). Briefly, 50µl of the cell suspension was added to 50µl lysis reagent A-100 and 50µl stabilizing reagent B (ChemoMetec A/S; Denmark). The solution was then loaded into a Nucleocassette (ChemoMetec A/S; Denmark) for the cell count. For solutions in which the initial cell count was too high to give an accurate reading, the cell suspension was diluted 1:10 using sterile saline and the analysis repeated.

2.3.3 Pulmonary Cell Differential Counts Following Particle Administration

Cytocentrifuge smears were prepared by adding 1×10^6 cells from the cell suspensions described above (Chapter 2.3.2) to 300 μ l saline containing 0.1% bovine serum albumen (BSA). Samples were centrifuged (300g, 3 min, room temperature) onto a microscope slide (Superfrost Plus, 75mm x 25mm. VWR International Ltd; Leicestershire, UK), and stained with Diff-Quick (Raymond A Lamb. London, UK) for differential cell counts to be assessed. 300 cells per slide were counted and the results expressed as total number of each cell type in the lung lavage by referring back to the original cell count (Duffin *et al.* 2001; Duffin *et al.* 2007). Macrophages were identified as large, circular cells with single-lobed nuclei, neutrophils were identified as smaller cells with multi-lobed nuclei (Figure 2.1).

Figure 2.1 - Differential Analysis of Pulmonary Inflammatory Cells.

Microscope image showing macrophages (large, circular, single lobed nuclei) and neutrophils (smaller, multi-lobed nuclei) after Diff-Quick staining. Image taken 6 hours after instillation of DEP, causing pulmonary inflammation (neutrophil influx). Magnification x400.



2.3.4 Measurement of Cell Death and/or Pulmonary Inflammation

Lactate dehydrogenase (LDH) is an enzyme released after cell death and lysis. It is also found in lung exudate, and is a marker of pulmonary inflammation. LDH levels taken from the BAL fluid were assessed using a cytotoxicity detection kit (LDH. Roche Diagnostics Ltd; Burgess Hill, UK), according to manufacturer's instructions. Cell pellet suspensions were stored briefly at 4°C before performing lactate dehydrogenase assays; assays were always performed on the same day as surgery. Briefly, 100µl of the resuspended cell pellet obtained from BAL fluid were added to working solution (100µl) in a 96-well plate. Positive control wells also received 5µl lysis solution, while negative control wells received deionised water instead of the cell suspension. The plate was read at 490nm with a 630nm reference filter (MRX plate reader. Dynex Technologies; Berlin, Germany), and LDH level was calculated as a percentage from positive and negative control values (Duffin *et al.* 2007).

2.3.5 Quantification of Protein Content in Bronchoalveolar Lavage Fluid

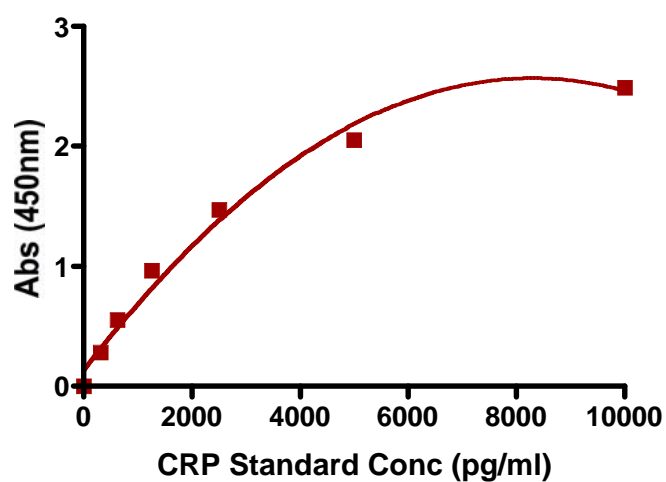
Protein content of BAL fluid provides another measure of pulmonary inflammation, as inflamed lungs allow the exudate influx, which increases protein levels. The total protein content of the resuspended cell pellet obtained from BAL fluid samples was measured using a bicinchoninic acid (BCA) protein assay (Thermo Scientific; Northumberland, UK), according to manufacturer's instructions. Resuspended cell pellet were stored briefly at 4°C before performing protein assays; assays were always performed on the same day as surgery. Briefly, BAL fluid (10µl) and BSA standards (10µl; 0.025 to 2mg/ml) were added to a 96-well plate. A 1:50 solution of copper (II) sulphate and BCA was prepared and added (190µl) to each well. The plate was incubated (37°C, 30 minutes), and then read at 570nm using a plate reader (MRX plate reader. Dynex Technologies; Berlin, Germany). The known standard concentrations were used to generate a standard curve from which sample protein concentrations were interpolated.

2.3.6 Measurement of Pro-Inflammatory Cytokines in Bronchoalveolar Lavage Fluid

BAL fluid was collected and stored at -80°C (as described in Chapter 2.1.3.1) for ELISA analysis for markers of inflammation including interleukin 6 (IL-6. DuoSet ELISA Rat IL-6 kit; R&D Systems; Abingdon, UK), C reactive protein (CRP. DuoSet ELISA Rat CRP kit; R&D Systems; Abingdon, UK) and tumour necrosis factor alpha (TNF α . DuoSet ELISA Rat TNF α kit; R&D Systems; Abingdon, UK). The standard protocol was the same for each ELISA; briefly, optically clear 96-well plates (R&D Systems; Abingdon, UK) were incubated at room temperature overnight with 100 μ l capture antibody at the required concentration for each assay. The plates were then washed 3 times with 400 μ l wash buffer (R&D Systems; Abingdon, UK) per well, before being blocked by adding 300 μ l reagent diluent (R&D Systems; Abingdon, UK) to each well, and incubating at room temperature (1 hour). The wash step was then repeated, and 100 μ l of samples or standards were added to each well in duplicate. The plate was covered and incubated at room temperature (2 hours). The wash step was then repeated, and 100 μ l detection antibody at the required concentration was added to each well. Once again the plate was covered and incubated at room temperature (2 hours). The wash step was repeated and 100 μ l of the working solution (Streptavidin-HRP) was added to each well. The plate was incubated in the dark at room temperature for 20 minutes before the final wash step. 100 μ l of substrate solution (1:1 mixture of H₂O₂ and Tetramethylbenzidine. R&D Systems; Abingdon, UK) was added to each well and the plate was incubated in the dark at room temperature (20 minutes) to allow for colour development. Stop solution (50 μ l; 2N H₂SO₄. R&D Systems; Abingdon, UK) was then added to each well and the plate was read at 450nm with the reference wavelength set at 550nm using a Triturus ELISA analyser (Grifols; Barcelona, Spain) and corresponding software. An example of a standard curve conducted for the CRP ELISA is shown (Figure 2.2).

Figure 2.2 - Standard Curve for C Reactive Protein ELISA.

Standard curve for CRP ELISA with a non-linear second order polynomial curve. Graph shows linearity up to 4000pg/ml and is very close to complete transection of both axes at zero. All sample values were situated in the linear part of the standard curve.



2.4 The Impact of Particulate Administration on Systemic Inflammation

Plasma samples collected from animal exposures were assessed for levels of IL-6, CRP and TNF α . Samples were stored and analysed using the same method used to analyse BAL fluid samples (Chapter 2.1.3.6).

2.5 The Impact of Particulate Administration on Endogenous Fibrinolytic and Clotting Pathways

The effect of particulate exposures on clotting systems was determined using flow cytometry to assess platelet-monocyte interactions and ELISAs to measure circulating concentrations of endogenous thrombolytic factors. Blood was collected from the rat abdominal aorta by syringe, and mixed with 3.8% sodium citrate solution (dilution factor 10:1). Fresh whole blood was used immediately for flow cytometry. Whole blood was centrifuged (1500g, 5 minutes, room temperature) to obtain plasma for ELISAs, which was separated into aliquots (0.5ml) and stored at -80°C until further analysis.

2.5.1 Impact of Particulate Administration on Platelet-Monocyte and Platelet-Platelet Interactions

In order to characterise platelet-monocyte and platelet-platelet aggregation following exposure to particulates, flow cytometric analysis was carried out to measure the percentage of cells positive for platelet and monocyte cell surface markers. Flow cytometry is a method of characterising cells based on how they fluoresce and scatter light as they are passed in suspension through a laser beam. Forward scatter (FS) of the laser can be used to provide information on the relative size of the cell, while side scatter (SS) of the laser provides information about the relative granularity (Ibrahim

& Van Den Engh. 2007). This method has been previously used to study platelet-monocyte aggregation (Tarnow *et al.* 2008). Fluorescent-conjugated antibodies can be used to detect specific cell surface markers; fluorescein isothiocyanate (FITC)-labelled anti-rat CD42d antibody was used to label platelets (detected in the FL1 channel; Sato *et al.* 2000), phycoerythrin (PE)-labeled hamster anti-rat CD61 was used to label monocytes (detected in the FL2 channel; Wiwanitkit. 2005). The FL1 and FL2 channels are photomultipliers and filters, used to detect light emitted from the fluorophores FITC (green light) and PE (orange light). These channels are able to detect light and also convert the strength of the light signal into a quantitative measure of labeled cells. PE-labelled hamster IgG1 κ was used as a negative isotype control in order to check for non-specific staining (All antibodies from BD Biosciences; Oxford, UK). Platelet-monocyte aggregates are defined as double positive for CD42d and CD61, platelet-platelet aggregates are defined as CD42d positive with a further shift in the FL1 channel away from the main group of single platelets. Blood was collected into 3.8% sodium citrate solution (dilution factor 10:1) from the abdominal aortae of rats exposed to particulates. Whole blood (30 μ l) was added to 30 μ l of diluted antibody (CD42d - 1/100 dilution in PBS, CD61 and isotype control 1/50 dilution in PBS), or 15 μ l of each antibody when double stains were performed. Samples were incubated at room temperature in the dark (20 min) before the addition of 500 μ l 1x FACS lysing solution (BD Biosciences; Oxford, UK), which causes lysis of red blood cells only. Samples were then incubated for a further 10 minutes before being analysed using flow cytometry (BD FACScan flow cytometer using CellQuest software and FlowJo software for analysis). A graph of the initial set-up showing forward scatter and side scatter can be seen below (Figure 2.3). Examples of FL1/FL2 scatter plots showing platelet-monocyte and platelet-platelet aggregation are also shown (Figure 2.4).

Figure 2.3 - Initial Set-Up and Gating of Whole Cell Population Via Flow Cytometry.

Forward scatter (FSC-H) vs. side scatter (SSC-H) plot taken from set up of flow cytometry experiments. Plot shows dead cells located with low forward scatter. A gate was set up (black square) to eliminate dead cells from further analysis, only cells located inside the gate were analysed for expression of fluorescent conjugated antibodies. Lymphocyte, neutrophil and monocyte populations are labelled.

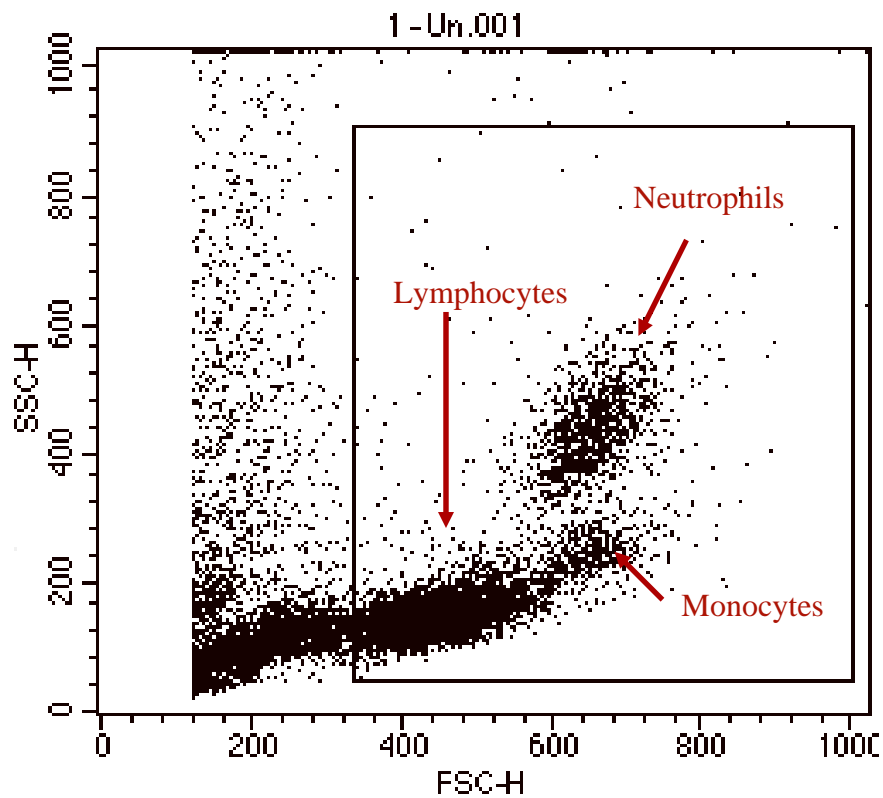
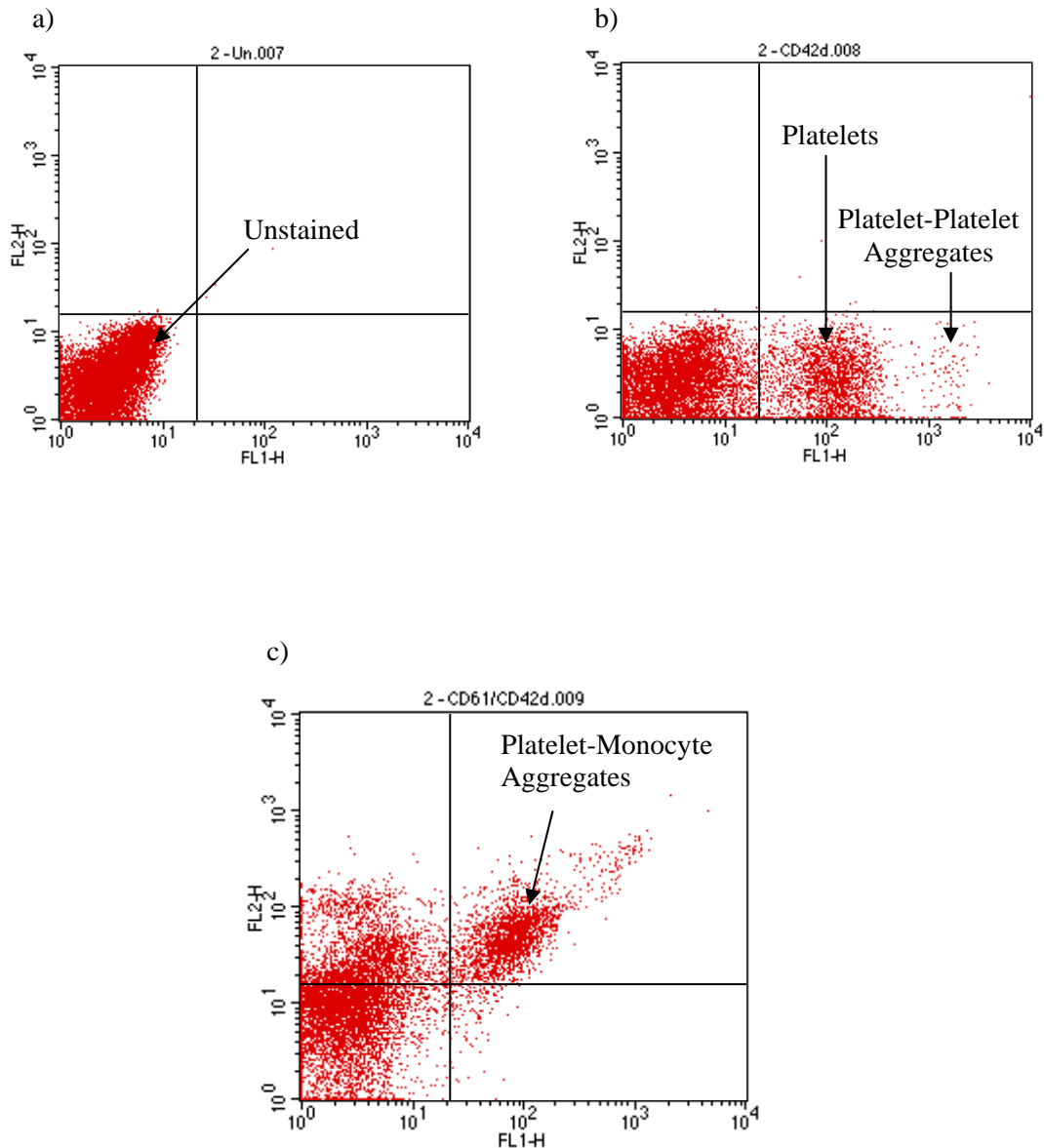


Figure 2.4 - Flow Cytometry Scatter Plots Showing Platelet-Monocyte and Platelet-Platelet Aggregation.

a) FL1/FL2 plot showing unstained control cells taken following instillation of saline. All cells are found in the double negative region of the plot. b) FL1/FL2 plot showing cells stained with fluorescein isothiocyanate (FITC)-labelled CD42d antibody following instillation of saline. Plot shows a large proportion of cells labelled with the CD42d antibody in the FL1 positive section, these cells are identified as platelets. Plot also shows a smaller subgroup further along the FL1 axis, which represents platelet-platelet aggregates. c) FL1-FL2 plot showing cells stained with both FITC-labelled CD42d antibody and phycoerythrin (PE)-labelled CD61 antibody following instillation of saline. Plot shows a large proportion of cells in the FL1 positive FL2 positive section. These cells are identified as platelet-monocyte aggregates.



2.5.2 Measurement of Markers of the Endogenous Thrombolytic System

In order to determine whether administration of particulate solutions altered endogenous fibrinolysis, plasma samples were assessed for levels of tissue plasminogen activator (t-PA), plasminogen activator inhibitor (PAI-1), fibrinogen and D dimer. Upon thrombus formation, fibrinogen is cleaved to form fibrin, which stabilises the clot. t-PA enzyme is released from endothelial cells in response to thrombus formation. t-PA breaks down inactive plasminogen to active plasmin, which in turn breaks down fibrin into fibrin degradation products and D-dimer. PAI-1 enzyme is also released from endothelial cells to inactivate t-PA and control the fibrinolytic process. Rat t-PA antigen, rat PAI-1 antigen and rat fibrinogen antigen ELISAs were obtained from Patricell Ltd. (Nottingham, UK). The standard protocol was the same for each ELISA; briefly, 100µl of samples and standards were added to duplicate wells on the plate provided in the kit. The plate was incubated at room temperature for 30 minutes agitated on a Belly Dancer (Stovall Life Sciences; Greensboro, NC, USA) at maximum speed. After incubation, the plate was washed 3 times with 300µl wash buffer (provided in the kits), and 100µl of reconstituted primary antibody was added to each well. The plate was incubated (30 min; room temperature on the Belly Dancer) before the wash step was repeated. Reconstituted secondary antibody (100µl) was then added to each well, the plate was incubated and washed again in the same manner. Substrate solution (100µl) was then added to each well and the plate was incubated (up to 15 min) to allow colour development. The reaction was then quenched by the addition of 50µl of stop solution (1N H₂SO₄), and absorbance of each sample was measured at 450nm (MRX plate reader. Dynex; Technologies, Berlin, Germany). D dimer ELISAs were performed by Pamela Dawson (Department of Haematology, Royal Infirmary of Edinburgh) using HemosIL D dimer HS reagents assessed using an ACL TOPS analyser (both from Beckman Coulter Ltd; Buckinghamshire, UK). Sample concentrations were calculated from the standard curves conducted with every assay.

2.6 Preparation of DEP Suspensions

Suspensions (1mg/ml) of DEP (SRM-2975; National Institute of Standards and Technology) were made up in endothelial cell culture medium (EGM-2 BulletKit Lonza Walkersville; Maryland, USA) without serum, containing 0.5mg/ml bovine serum albumen (BSA) and prepared using a probe sonicator (Status Homogenisers. Philip Harris Scientific; US70, Lichfield, UK) at 70% power for 5 minutes, to ensure dispersion. Suspensions were then centrifuged (500g for 1 hour) to remove large particle aggregates

2.6.1 Analysis of Particle Size in DEP Suspensions

To determine particle sizes 1:50 dilutions of the DEP suspensions were measured using a particle size analyser (90 Plus particle size analyser. Brookhaven Instruments Corporation; NY, USA), which uses laser-scatter to calculate mean particle diameter (O'Brien *et al.* 2006). The settings for these experiments were as follows; each run lasted 1 minute with a total of 10 runs per sample, laser angle for light scatter was fixed at 90°, laser wavelength was 659nm, the refractive index was set at 1.5 and the dust cut-off filter was set at 30µm (the machine is accurate between 3nm and 3µm).

2.6.2 Absorbance Spectra of DEP Suspensions (Indirect Measure of Suspension Concentrations)

In order to provide an estimate of particle concentration remaining in suspension during the initial testing phase, DEP suspensions were diluted to a 1:50 concentration with dH₂O and analysed in a spectrophotometer (SmartSpec 3000. Bio-Rad; Hertfordshire, UK) measuring absorbance at 500nm.

2.6.3 Final Analysis of DEP Suspension Concentrations

The exact concentration of DEP in this dispersant after centrifugation was investigated by spinning 1ml samples of the DEP suspension in a heated vacuum centrifuge overnight, along with 1ml samples of serum free medium + 0.5mg/ml BSA. The dry weights of the serum free medium + BSA samples were subtracted from the dry weights of the DEP samples to give the mass of DEP in a 1ml suspension. This was then used to calculate DEP concentrations in all future experiments.

2.7 Endothelial Cell Culture

Human umbilical vein endothelial cells (HUVECs; Promocell; Heidelberg, Germany) were cultured at 37°C in 75cm³ flasks using endothelial cell culture medium (EGM-2 BulletKit - basal medium containing human epidermal growth factor, hydrocortisone, gentamicin, amphotericin B, fetal bovine serum, vascular endothelial growth factor, human fibroblast growth factor B, insulin-like growth factor 1, ascorbic acid and heparin - Lonza Walkersville; Maryland, USA). When cells had grown to confluence they were passaged by first removing the culture medium and then washing the cells twice with 3ml HEPES-buffered saline solution (Reagent Pack Subculture Kit - Lonza Walkersville; Maryland, USA). Cells were then incubated with 3ml trypsin/EDTA solution (Reagent Pack Subculture Kit - Lonza Walkersville; Maryland, USA) at 37°C for 5 minutes, and the sides of the flask were gently tapped to lift cells from the culture surface. 5ml trypsin neutralising solution (Reagent Pack Subculture Kit - Lonza Walkersville; Maryland, USA) was then added to the cells to prevent cell lysis. The cell suspension was then split equally between four new flasks, 15ml culture medium was added and the flasks were incubated at 37°C. Cells up to passage 6 (P6) were used for further experiments.

2.7.1 Exposure of Human Umbilical Vein Endothelial Cells (HUVECs) to DEP Suspensions

HUVECs were exposed to DEP in 2 separate experiments. The first was designed to investigate the effects of DEP on constitutive release of fibrinolytic factors, by exposing HUVECs to DEP only. HUVECs were washed twice with HEPES-buffered saline solution, and full growth medium was replaced with serum free medium containing DEP (10, 20, 50, 100 or 150µg/ml) in serum free medium plus BSA (0.5mg/ml). Controls were exposed to serum free medium alone. Cells were incubated under these conditions for 2, 6, 16 or 24 hours. The second experiment was designed to investigate the effects of DEP on stimulated release of fibrinolytic factors. In these experiments, HUVECs were first incubated with either 100µg/ml DEP (made up in serum free culture medium + 0.5mg/ml BSA, added to 15mls serum free culture medium for exposure) or serum free medium alone for 16 hours. Cells were then washed and exposed to a range of thrombin concentrations (0.1, 0.5 and 1U/ml in serum free culture medium with serum free culture medium used as a second control) for 24 hours. Following incubation, cell supernatants and cellular RNA were isolated and stored at -80°C for later analysis.

2.7.2 Assessment of Cell Viability

In cells undergoing experimental exposures to DEP, viability was assessed using two complementary techniques:

2.7.2.1 Trypan Blue Exclusion

HUVECS (200,000 cells/well in 2ml growth medium) were plated onto 6 well plates and incubated (2 hours, 37°C) to allow adhesion to the well surface. These were then incubated (under standard culture conditions) with DEP solutions (20 and 60µg/ml;

15ml final volume in serum free cell culture medium) for up to 24 hours. Control wells contained dispersant in culture medium without DEP. After the appropriate exposure time, HUVECs were photographed and assessed for viability using Trypan Blue exclusion (Cook and Michell, 1989). Briefly, the HUVECs were washed and trypsinised and an aliquot (10µl) of the cell suspension was mixed with 90µl trypan blue (Sigma Aldrich; Dorset, UK). Live cells actively exclude the stain and appear white when examined microscopically. Dead cells cannot exclude the stain and, therefore, appear dark blue. Live and dead cells were counted using a haemocytometer; the number of dead cells was expressed as a percentage of total cells counted.

2.7.2.2 Lactate Dehydrogenase Release

In cultured cells the LDH assay is only a marker of cell death and cytotoxicity, as opposed to experiments using BAL fluid where LDH can also detect pulmonary inflammation. To measure lactate dehydrogenase (LDH) release HUVECs (50,000 cells/well in 100µl cell culture medium) were seeded on 96 well plates and incubated (2 hours, 37°C) to allow adhesion to the well surface. Cells were incubated (under standard conditions for up to 24 hours), with positive cytotoxic controls, hydrogen peroxide (1µM - 1mM) and Tween 20 (0.1% - 50%), to provide high control levels of cell death. Test exposures were achieved by incubating cells (at 37°C for 2, 6, 16 and 24 hours) with DEP (at 10, 20, 40, 60 and 80µg/ml in serum free medium containing 0.5mg/ml BSA) or vehicle. LDH release was determined using a colorimetric assay (cytotoxicity detection, LDH assay kit; Roche Diagnostics). Briefly, cells were incubated with DEP, hydrogen peroxide, Tween 20 or serum free medium for the required exposure times in 96 well plates. Microplates were centrifuged at 250g for 10 minutes and supernatant (100µl) was carefully transferred from each well into corresponding wells on a second optically clear, flat-bottomed 96 well microplate. Reaction solution was a mixture of catalyst solution (diaphorase/NAD⁺ mixture) and dye solution (iodotetrazolium chloride and sodium

lactate); 100µl of reaction solution was added to each well and the plate was incubated at room temperature(30 minutes) in the dark. The plate was assessed by measuring absorbance at 490nm (MRX plate reader. Dynatech Laboratories; VA, USA). Cytotoxicity was calculated as a percentage of maximum, as determined from control wells containing no cells (to measure background absorbance at each of the DEP concentrations) and positive control wells (containing cells treated with 50% detergent to rupture cell membranes resulting in total cell lysis).

2.7.3 Measurement of t-PA and PAI-1 Released by HUVECs

The influence of experimental manipulations on the endogenous fibrinolytic system in HUVECs was assessed by measuring release of key components of this system into the culture medium. The effect of DEP on stimulated t-PA and PAI-1 release was assessed by incubating HUVECs in serum free medium with DEP (100µg/ml for 16 hours) or vehicle. DEP was then removed by washing with HEPES buffer, and replaced with serum free medium containing thrombin (0.1, 0.5 or 1 U/ml: 24 hour exposure). Following both incubations, cell supernatants and cellular RNA were isolated and stored at -80°C for subsequent analysis using t-PA ELISA and RT-PCR (as described in Chapter 2.7.3.1), respectively. In order to stimulate t-PA and PAI-1 release HUVECs were incubated with 0, 0.1, 0.5 or 1 U/ml thrombin (Sigma Aldrich; Dorset, UK) for 6 or 24 hours.

2.7.3.1 t-PA ELISA

Activity and antigen concentrations of t-PA were measured in cell supernatants using a commercially available ELISA (t-PA Combi Actibind ELISA kit. Technoclone Ltd; Surrey, UK.), according to manufacturer's instructions. Briefly, sample supernatants were diluted 1:4 with incubation buffer and 100µl of each sample and standards were added in triplicate to separate wells of the test microplate for overnight incubation at 4°C. The plate was then washed 3 times and 200µl of

detection mixture was added to each well; the plate was incubated (37°C, 1 hour) and absorbance was measured using a plate reader (MRX plate reader. Dynatech Laboratories; VA, USA) at 405nm to assess t-PA activity (0.125-10U/ml). The plate was then washed 3 times before diluted horseradish peroxidase (POX) conjugated monoclonal anti-t-PA (100µl) antibody was added to each well, the plate was then incubated for 1 hour at 37°C. The plate was washed a further 3 times, 100µl of TMB substrate was added to all wells and the plate was incubated at room temperature for 10 minutes before the addition of 100µl of stop solution. Absorbance was then assessed using a plate-reader at 450nm to assess t-PA antigen levels in reference to readings from the standards (0.125-30ng/ml).

2.7.3.2 PAI-1 ELISA

Supernatants taken from HUVECs exposed to DEP solutions or vehicle were assessed for PAI-1 activity using a commercially-available ELISA (TECHNOZYM® PAI-1 Actibind® ELISA Kit. Technoclone Ltd; Surrey, UK), according to manufacturer's instructions. Briefly, 25µl of controls and sample plasma were added in triplicate to separate wells of the test microplate along with 75µl of diluted POX conjugated monoclonal anti-PAI-1 antibody working solution. The plate was incubated at 37°C for 45 minutes. It was then washed 3 times before adding substrate solution (100µl) to each well; the plate was then incubated (15 min, room temperature) before adding stop solution (100µl). Absorbance was then assessed using a plate reader (MRX plate reader. Dynatech Laboratories; VA, USA) at 450nm to assess PAI-1 activity in reference to readings from the standards (0.3-61.1U/ml).

2.7.4 Molecular Analysis of HUVECs

Qualitative molecular analysis of HUVECs was performed using RT-PCR to confirm the presence of markers of endothelial cell phenotype. Quantitation of expression of

components of the fibrinolytic system was achieved using quantitative real time PCR.

2.7.4.1 Reverse Transcription Polymerase Chain Reaction for Effects of DEP Exposure on the Thrombolytic System

2.7.4.1.1 RNA Extraction

Cultured cells were trypsinised and centrifuged (1000rpm, 5 min) and the supernatant removed and discarded. RNA extraction was achieved using the TRIzol extraction method. Briefly, cells were incubated (room temperature, 5 minutes) in TRIzol (1ml, Invitrogen), then mixed with chloroform (0.2ml, Fisher Scientific; Loughborough, UK) and incubated (room temperature) for a further 3 minutes. Samples were centrifuged (12,000g, 15 minutes, 4°C) to separate the solid and aqueous phases. The aqueous phase was transferred to a fresh Eppendorf tube and isopropanol (0.5ml, Fisher Scientific; Loughborough, UK), added to precipitate the RNA. Samples were incubated (room temperature, 10 min) and centrifuged (12,000g, 10 minutes, 4°C), the supernatant was removed and discarded, and the RNA pellet was washed with cold (4°C) 75% ethanol (1ml, Fisher Scientific; Loughborough, UK). Samples were vortexed and centrifuged (7,500g, 5 minutes, 4°C), the supernatant discarded and the RNA pellets allowed to air dry (room temperature, 5 minutes). Pellets were then resuspended in autoclaved 0.1% diethylpyrocarbonate (DEPC)-treated water (50µl) by incubating at 60°C for 10 minutes.

2.7.4.1.2 RNA Integrity

RNA concentration was determined using a GeneQuant spectrophotometer reading at 320nm. The integrity of the extracted RNA was verified by denaturing agarose gel electrophoresis. A small (50ml) gel tray and comb were cleaned and treated with

'RNase Zap' (Ambion International; Cambridgeshire, UK). The 1.2% agarose gel was prepared by adding 0.6 g agarose (Cambrex; NJ, USA) to 44 ml DEPC water and boiling in a microwave (30 seconds). The mixture was left to cool slightly, then formaldehyde (1ml, Sigma-Aldrich; Dorset, UK), 5 ml autoclaved 10x 4-morpholinepropanesulfonic acid (MOPS) solution (0.4 M MOPS, 0.1 M Sodium acetate, 10 mM EDTA; pH 7; Sigma Aldrich; Dorset, UK) and 4µl dilution of ethidium bromide (Sigma-Aldrich; Dorset, UK) were added and mixed in a fume hood. The mixture was poured into the tray and left to set. RNA samples (2µl) were denatured by mixing with 2.5µl formaldehyde, 2.5µl 10x MOPS, 10µl deionised formamide (Sigma-Aldrich; Dorset, UK) and 8µl DEPC water and incubating at 65°C for 15 minutes. Loading buffer (2µl) was added to each of the samples and 10µl of each sample mixture was then loaded into separate wells of the agarose gel and run in 1x MOPS for 40 minutes at 150v. RNA was then visualised on a UV trans-illuminator at 254nm and photographed using a Mitsubishi P93 video printer.

2.7.4.1.3 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Reverse Transcription PCR was conducted to show RNA expression in HUVECs. RT-PCR was conducted using primers for Tie2 (an endothelial cell marker) to show that HUVECs were still of endothelial lineage up to P6 (Partanen *et al.* 1992). Reverse transcription of extracted RNA was performed using a Promega A3500 RT system (Promega Corporation; Hampshire, UK). RNA (1µg/ml) was added to the Promega Mastermix (containing 10x Buffer, MgCl₂, Oligo nucleotides, dNTPs and RNase inhibitor at a ratio of 2:4:1:2:1). Reverse transcriptase enzyme was added and samples were incubated at 42°C for 30 minutes, 99°C for 5 min and 4°C for 10 minutes in an Eppendorf 5331 thermal cycler. At this time two negative controls were added; one containing no reverse transcriptase enzyme, and a second containing no RNA. PCR was performed using a Promega M5661 Taq Bead Hot Start Polymerase kit. cDNA samples were added to the Promega Mastermix (containing 10x Buffer, MgCl₂, dNTPs, forward primer, reverse primer and nuclease free H₂O at

a ratio of 5:3:1:2:2:32). Samples were run in an Eppendorf 5331 thermal cycler at 95°C for 45 seconds, then 35 cycles of 90°C for 45 seconds, 60°C for 30 seconds, 72°C for 90 seconds, 72°C for 5 minutes and finally 4°C for 10 minutes. Samples were run on a 1.2% agarose gel along with a 100bp DNA ladder (Promega Corporation; Hampshire, UK) and visualised using a UV trans-illuminator at 254nm. Expression of t-PA, PAI-1 and GAPDH were assessed via RT-PCR following the RNA extraction method discussed above, following incubation with DEP. RT-PCR was conducted using primers for t-PA, PAI-1 and GAPDH; primer sequences are shown (Table 2.1).

2.7.4.2 Quantitative Real-Time Polymerase Chain Reaction

RNA was extracted from HUVECs exposed to DEP as described previously (Chapter 2.7.3.1.1), reverse transcribed to cDNA and stored at -80°C until real-time PCR was performed. Real time primers (shown in Table 5.2, Invitrogen; Paisley, UK) were diluted to 200pM using TE (Tris EDTA buffer solution. pH 8.0. Fluka BioChemika; Switzerland). Each primer (0.1µl) was added to 0.1µl of the corresponding fluorescent probe, 2.7µl RNase free H₂O and 5µl Roche probe master (both from LightCycler 480 probes master kit; Roche; Burgess Hill, UK), providing an 8µl Mastermix that was added to each well. 2µl of cDNA (previously diluted 1:40 using RNase free H₂O) was then added to each corresponding test well. Probes were obtained from the Roche Universal Probe Library, and primers were designed using the Roche UPL Design Centre (Roche; Burgess Hill, UK) using the species-specific gene and nucleotide sequence. Standard curves were conducted for each gene tested, starting at a 1:8 cDNA dilution with 7 further 1:2 dilutions. RNase free H₂O was also tested as a negative control. Samples were analysed using a Roche LightCycler 480 and corresponding software, and by comparing the unknown samples with standard curves for each set of primers. Results from housekeeping genes (GAPDH, β-actin) were used to normalise the data and show changes in the test genes (t-PA, PAI-1).

Table 2.1 - Reverse Transcription Polymerase Chain Reaction Primers.

RT-PCR primer sequences and band sizes for endothelial tyrosine kinase receptor (Tie2), tissue plasminogen activator (t-PA), plasminogen activator inhibitor (PAI-1) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

	Forward Primer	Reverse Primer	Band Size
Tie2	TCA CTC CAG TAT CAG CTC AAG GG	CAG CTG GTT CTT CCC TCA CGT T	300bp
t-PA	ATC TTT GCC AAG CAC AGG AGG TCG	CTT CAG CCG CTC CGA ATA GAA AGG	410bp
PAI-1	AGC ACG GTC AAG CAA GTG GAC T	GGC AGT TCC AGG ATG TCG TAG TAA	325bp
GAPDH	CCA CCC ATG GCA AAT TCC ATG GCA	TCT AGA CGG CAG GTC AGG TCC ACC	600bp

Table 2.2 - Real Time Polymerase Chain Reaction Primers.

Real-time PCR primer sequences for tissue plasminogen activator (t-PA), plasminogen activator inhibitor (PAI-1), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β Actin.

	Forward Primer	Reverse Primer
t-PA	CGG GTG GAA TAT TGC TGC T	CTT GGC TGG CTG CAA CTT
PAI-1	CTC CTG GTT CTG CCC AAG T	CAG GTT CTC TAG GGG CTT CC
GAPDH	GCT AGG GAC GGC CTG AAG	GCC CAA TAC GCC AAA TCC
β Actin	CCA ACC GCG AGA AGA TGA	TCC ATC ACG ATG CCA GTG

2.8 Statistics

Data are expressed as mean \pm SEM. Results were analysed using GraphPad Prism software. Comparison of 2 groups was performed using an unpaired two-tailed t-test. Comparison of 3 or more groups was performed using a one-way ANOVA followed by a Tukey's multiple comparison post-test. Comparison of 2 groups at 3 different time-points was achieved using a two-way ANOVA with a Bonferroni post-test. Significance was assumed when $P < 0.05$. Non-statistically significant data trends were discussed when $P < 0.1$ to allow consideration of experimental power.

Chapter 3

Development of an In Vivo model of Thrombus Formation

3.1 Introduction

Animal models are useful tools for improving our understanding of the regulation of arterial thrombosis *in vivo*. In order to assess the effects of diesel exhaust particulates (DEP) on thrombus formation *in vivo*, a suitable experimental model was required. None of the currently used experimental models were established in the department, so the introduction of a suitable model required method development. The model required for the following experiments would ideally replicate components of the clotting response in human atherothrombosis; for example, endothelial dysfunction/removal, arterial occlusion and arterial damage leading to the formation of a platelet-rich thrombus (Watson *et al.* 1985; Kurz *et al.* 1990; Denis *et al.* 1998; Farrehi *et al.* 1998; Furie & Furie. 2005). As discussed previously (see Chapter 1.2.3.4), several models of arterial thrombosis have been developed. These are commonly based on the induction of injury to the artery wall followed by either measurement of blood flow or of thrombus development. The main differences between models are in the target blood vessel and in the techniques used to cause the initial injury and to measure clot formation.

The Folts model of arterial thrombosis, which induces both endothelial damage and arterial stenosis in order to promote thrombus formation, is widely regarded as the pharmaceutical industry 'gold standard' technique. It was first developed in anaesthetised dogs, and has since been adapted for use in small animals such as mice (Sturgeon *et al.* 2006), rats (Daykin *et al.* 2006; Sturgeon *et al.* 2006) and rabbits (Marret *et al.* 2004; Sturgeon *et al.* 2006). The adapted Folts models induce 50% or 60% stenosis of the carotid artery and this stenosed section is then injured mechanically (by repeated pinching with a pair of forceps). This injury causes de-endothelialisation leading to occlusive thrombus formation. Embolisation of the thrombus re-opens the vessel and re-starts the clotting procedure (Sturgeon *et al.* 2006; Daykin *et al.* 2006), and when repeated produces cyclic flow reductions (CFRs); the number of CFRs over a set period of time give a quantitative measure of thrombogenicity.

An alternative approach is to injure the endothelium by application of ferric chloride (FeCl_3) to the adventitial surface of an artery damages (Kurz *et al.* 1990). This typically leads to complete vessel occlusion. Quantification is achieved by timing the period from application of FeCl_3 to complete cessation of blood flow in the target artery. This model has also been widely used in small rodents and has the advantage of being relatively straightforward and reproducible. The third most commonly used technique for inducing thrombosis in rodent arteries induces injury by illumination of a photo-reactive substance (such as Rose Bengal) resulting in the local production of damaging reactive oxygen species. Formation of thrombus is then measured by reduction of blood flow as measured via flow probe. However, whilst the use of FeCl_3 or photosensitive dyes induces thrombus formation through endothelial damage, they do not produce arterial stenosis. Whilst this could appear to be a shortcoming it should be noted that outward remodelling often occurs in atherosclerosis, preventing lumen narrowing (Jackson *et al.* 2007).

In addition to induction of arterial thrombosis, alterations in thrombosis in rats and mice can be assessed by measuring tail tip bleeding time (Elg *et al.* 1998; Sato *et al.* 1998; Tanaka *et al.* 1998; Wang & Xu. 2004; Daykin *et al.* 2006). Use of this model would help to distinguish the effects of DEP on haemostasis from the effects on platelet activation and aggregation, activation of the coagulation cascade and fibrin deposition. It is also a relatively straightforward model that could be performed in animals used for induction of arterial thrombosis.

Experimental thrombosis has been induced both in arteries and in veins in rodents (Nemmar *et al.* 2002; Nemmar *et al.* 2003; Day *et al.* 2004; Rumbaut *et al.* 2005; Daykin *et al.* 2006) and DEP has been shown to increase thrombosis in both types of vessels (Nemmar *et al.* 2003). For the purposes of this thesis, however, arteries were studied, as the disease of interest, atherothrombosis, affects predominantly medium-sized muscular arteries (Soldatos & Cooper. 2006). Studies in larger mammals (eg dogs) often target the coronary arteries (Folts *et al.* 1982), but those in small animals generally use the carotid artery. This is necessary due to the small size and difficult accessibility of the coronary artery in small animals, but it also produces more

reliable and reproducible results by avoiding the haemostatic disturbance associated with open-chest surgery.

Finally, the choice of animal used in these experiments was taken into consideration. Both rats and mice have been used previously to study the effects of pollution (Yoshino *et al.* 1999; Ito *et al.* 2000; Hirano *et al.* 2003; Wold *et al.* 2006; Arimoto *et al.* 2007; Yokota *et al.* 2008; Nemmar *et al.* 2008; Jaspers *et al.* 2009; Nemmar *et al.* 2009). These animals can be easily exposed to DEP in a variety of ways, for example, intratracheal instillation, inhalation or intravenous injection (Nemmar *et al.* 2002; Nemmar *et al.* 2003; Nemmar *et al.* 2004; Yokota *et al.* 2005; Wold *et al.* 2006; Nemmar *et al.* 2008). Mice are desirable because of the possible use of transgenics to clarify the mechanisms at work. However, their small size does present a problem for some of the previously described models, such as the FeCl₃ model (Kurz *et al.* 1990); in such cases rats are a useful alternative and have been used extensively to study the effects of pollution.

3.1.1 Hypothesis

The work described in this chapter addressed the hypothesis that it was possible to introduce a reproducible model of *in vivo* thrombus development in experimental animals, in order to study the effects of DEP.

3.1.2 Aims

The aims of the work described in this chapter were:

- i. to introduce a suitable *in vivo* model of reproducible thrombosis
- ii. to perform a pilot study to confirm that this model was suitable for testing the effects of diesel exhaust particulate on thrombus formation.

3.2 Methods

3.2.1 Animals

Male C57 Black 6 mice (aged 70-77 days) and male Wistar rats (100-125g) were obtained from Charles River Laboratories (Wilmington, MA, USA). All animals were housed for at least one week, with constant access to food and water and a 12 hour light/dark cycle, before use in the following experiments.

3.2.2 Development of Models of *In Vivo* Thrombosis

3.2.2.1 The Folts Model of Arterial Thrombosis

The Folts model of arterial thrombosis (Folts *et al.* 1982; Sturgeon *et al.* 2006) was attempted first in mice, and, subsequently, in rats. Animals were anaesthetised by inhalation of isoflurane (Merial, Essex, UK), body temperature was maintained at 37°C using a heat blanket, and hair was shaved from the anterior surface of the neck prior to surgery. The left carotid artery was then isolated by blunt dissection and blood flow through the carotid artery was measured by placement of an ultrasonic flow probe (Transonic Systems Inc. Maastricht, Netherlands). Waxed 6-0 Dysilk thread (Dyneke Pty Ltd. South Australia) was then tied around the carotid artery in 2 places, roughly 5mm apart, distal to the flow probe. These ligatures were tightened to create a 50% reduction in blood flow (as measured by the flow probe). The area between the ligatures was then crushed with forceps to damage the endothelial layer. Crushing was carried out in sets of five pinches of the artery, with this protocol repeated up to five times, as required. When effective, this procedure should produce a blood clot in the affected area, which reduces blood flow to zero. After 20 seconds in mice, and 30 seconds in rats, the clot is embolised by pinching the vessel with forceps. This restores blood flow until a new clot begins to form. The repeated reductions in blood flow as new clots form are known as cyclic flow reductions (CFRs). The number of CFRs in a 30 minute period are then recorded.

3.2.2.2 Ferric Chloride-Mediated Induction of Thrombosis

Induction of arterial thrombus by direct application of FeCl_3 (Kurz *et al.* 1990) was attempted using male Wistar rats (body weight 175-275g). Rats were anaesthetised, the left carotid artery exposed and carotid artery flow measured by emplacement of a flow probe, as described for the Folts Model (Chapter 3.2.2.1). FeCl_3 was applied topically to the carotid artery; this was achieved using a piece of plastic tubing (2mm internal diameter) approximately 3mm in length. The tubing was cut open and a strip of Whatman filter paper (No 5) was placed inside and folded to create a double layer. The tubing and filter paper were then dipped in FeCl_3 solution (concentration range 5-40%. BDH. Poole, UK) and placed around the carotid artery. Care was taken to ensure full contact between the filter paper and the artery wall. The FeCl_3 solution was left in contact with the artery for 10 minutes before being carefully removed. Blood flow through the artery was recorded and timing was stopped when blood flow reached 0ml/min. Preliminary experiments were performed to determine the optimum concentration of FeCl_3 for induction of thrombosis. All subsequent experiments were performed using this concentration (20% w/v).

3.2.2.3 Time to Arrest of Bleeding

In all animals, the time to arrest of tail tip bleeding (Tanaka *et al.* 1998) was assessed once total occlusion of the carotid artery had been achieved. The tail tip (5mm) was removed using a scalpel and cleaned every 10 seconds using a swab (Medical Wire and Equipment Company). Arrest of bleeding was considered to be the time when no blood was transferred from the tail to the swab.

3.2.3 Pilot Investigation into Effects of DEP on Thrombus Formation

DEP solutions (1mg/ml) were prepared in sterile 0.9% saline and sonicated (Status Homogenisers. Probe from Philip Harris Scientific, US70; Lichfield, UK) at 70% power for 5 minutes. DEP working solutions were then prepared to a final volume of 0.1ml with sterile 0.9% saline.

Male Wistar rats (body weight 175-275g) were prepared for surgery as described above (Chapter 3.2.2.2) and the left carotid artery exposed. The left jugular vein was isolated by blunt dissection and cannulated to allow bolus injection of DEP solutions (0.1ml) or vehicle (0.1ml; 0.9% saline). The rat was maintained under anaesthesia for 2 hours after injection. At the end of this period, FeCl₃ (20%) was applied to the left carotid artery, as described (Chapter 3.2.2.2), to induce clot development, and time to occlusion was recorded. Once the carotid artery had occluded, time to arrest of bleeding was measured using the tail tip technique (Chapter 3.2.2.3). Initial investigations used a dose of 500µg/kg DEP (Nemmar *at al*, 2003). However, during these preliminary experiments the animals developed breathing difficulties and died approximately 1 hour after injection. The dose was then reduced to 250µg/kg DEP; animals receiving this dose survived the 2 hour exposure time before induction of thrombosis, and so this concentration was used for all further infusion experiments.

3.2.4 Histology

Carotid arteries were taken from experimental animals after induction of thrombosis using either the Folts model or FeCl₃ application. Arteries were fixed in formalin for 24 hours, before being dehydrated in ethanol, processed and embedded in paraffin blocks. Blocks were later sectioned (4mm) and stained using the standard haematoxylin and eosin (H&E) method.

3.2.5 Statistics

All data are expressed as mean \pm SEM. The impact of exposure to DEP on FeCl₃-induced thrombosis and time to arrest of tail tip bleeding was assessed by comparing data from DEP- and vehicle-treated groups using an unpaired two-tailed t-test.

3.3 Results

3.3.1 Development of the Folts Model of Arterial Thrombosis

Initial experiments encountered methodological difficulties in applying the Folts model to mice. Blood flow through the artery could be successfully measured, but the small size of the carotid arteries in mice meant that it was not possible to accurately reduce blood flow by 50% and crush injury did not produce an occlusive thrombus (animals used and experimental outcome outlined in Table 3.1).

As a consequence of the problems experienced applying the Folts model to mice, rats were used in all subsequent experiments. Using rats, blood flow data logs taken during development of the Folts model confirmed that it was possible to reduce blood flow by approximately 50% by tightening the silk ligatures (Figure 3.1a). However, despite repeated attempts, mechanical damage to the exposed artery did not produce a completely occlusive thrombus that could then be embolised by agitating the artery (Figure 3.1b). Sections of carotid arteries were taken from rats after the Folts model had been applied and stained with haematoxylin and eosin. Typically no thrombus would form during this experiment (Figure 3.2a). On only one occasion a completely occlusive thrombus did form (Figure 3.2b), but it was not possible to embolise this thrombus by agitating the artery.

Table 3.1 - Experimental Outcomes of Folts Model Experiments.

Animals used and experimental outcomes of the Folts model experiments are outlined in the table below.

Animal	Outcome
Mouse 1	Sutures tightened (slip knot), blood flow reduced
Mouse 2	Sutures tightened (slip knot), blood flow initially reduced but sutures worked loose, no thrombus formation
Mouse 3	Sutures tightened (half hitch), 50% stenosis achieved, artery damaged by pinching, no thrombus formation.
Rat 1	Sutures tightened (half hitch) and secured in place using bulldog clips, artery damaged by pinching, no thrombus formation.
Rat 2	Sutures tightened (half hitch) and secured in place using bulldog clips, artery damaged by pinching, no thrombus formation.
Rat 3	Sutures tightened (half hitch) and secured in place using bulldog clips, artery damaged by pinching, occlusive thrombus formation but thrombus could not be fully embolised.

Figure 3.1 - Blood Flow Traces from Folts Model Experiments.

a) Blood flow trace showing successful reduction in blood flow (by 50%) in a rat carotid artery. b) Blood flow trace of a partially successful Folts model experiment in a rat; the beginning of the trace shows low blood flow due to pinching the artery to destroy the endothelium. Flow is then restored until an occlusive thrombus begins to form, reducing blood flow to almost zero. The artery was then agitated to embolise the thrombus. However blood flow was not restored to its original rate, despite repeated attempts. In both traces, time 0 indicated the beginning of the test and included initial set-up. Specific time points have been selected to show specified points of the test.

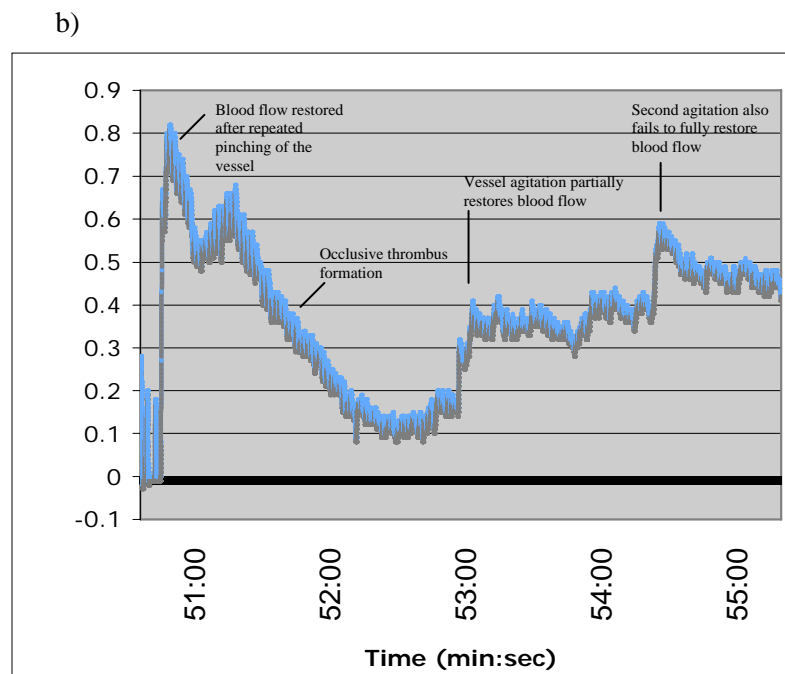
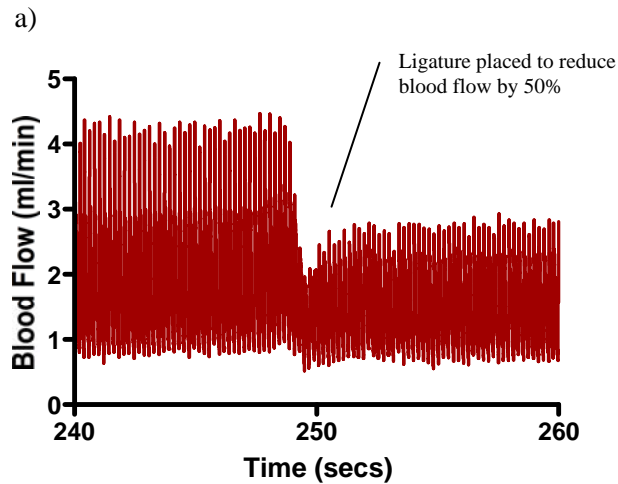
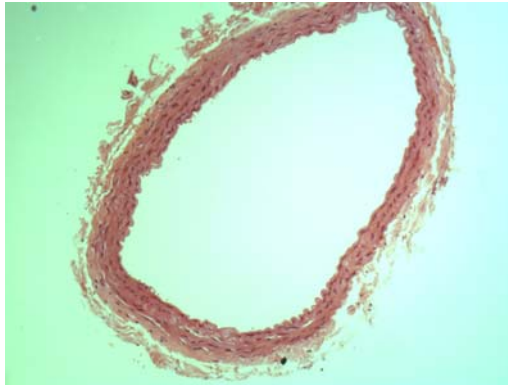


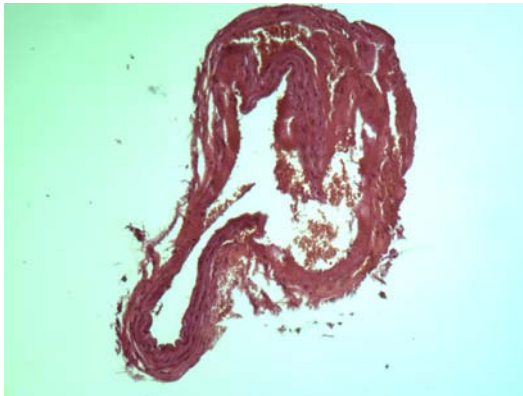
Figure 3.2 - H&E Staining of Carotid Arteries Following Application of the Folts Model.

a) Uninjured section of rat carotid artery. b) Unsuccessful attempt at thrombus formation; vessel wall is damaged due to stenosis and repeated pinching. c) Fully occlusive thrombus that could not be removed by agitating the artery. All stained using the H&E method. Magnification x100.

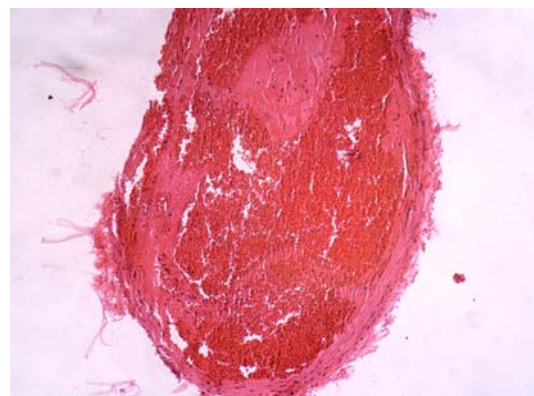
a)



b)



c)



3.3.2 Development of the Ferric Chloride Model of Arterial Thrombosis

Initial experiments were designed to determine the correct concentration of FeCl_3 to use in future experiments. Previous studies had used concentrations ranging from 10-70% (Denis *et al.* 1998; Kurz *et al.* 1990; Radomski *et al.* 2005; Wang & Xu; 2005). Initially a 40% solution of FeCl_3 was used, but this caused complete vessel occlusion in approximately 2.5 minutes. It was felt that this was too short a time to accurately detect changes between experimental groups, so a 20% solution was tried (Figure 3.3). This concentration typically produced complete vessel occlusion in approximately 15 minutes with a high degree of consistency (coefficient of variation = 0.167). It was decided that this concentration of ferric chloride would be used for all future experiments. 10% and 5% FeCl_3 solutions were also examined, but these solutions failed to produce complete vessel occlusion within 1 hour (Results of preliminary experiments into FeCl_3 concentration are shown in Table 3.2). Sections of carotid arteries exposed to FeCl_3 were stained with haematoxylin and eosin (Figure 3.4). In these pictures the occlusive thrombus is clearly visible, along with a dark ring around the inside of the artery where the FeCl_3 solution has collected and destroyed the endothelium.

3.3.3 Pilot Investigation into Effects of DEP on Thrombus Formation

The results of the pilot experiment showed that intravenous infusion of DEP (250 $\mu\text{g/kg}$) significantly reduced the time to vessel occlusion following administration of 20% FeCl_3 solution from 17.89 ± 0.67 mins (saline) to 15.66 ± 0.70 mins (DEP. $P=0.044$; $n=6$. Figure 3.5a). This result was mirrored by a similar reduction in tail tip bleeding time following DEP administration from 15.61 ± 1.41 mins (saline) to 11.25 ± 0.93 mins (DEP. $P=0.027$; $n=6$. Figure 3.5b).

Table 3.2 - Experimental Outcomes of Preliminary Experiments Investigating FeCl₃ Concentration.

Effects of FeCl₃ concentration on thrombus formation. Where thrombus formation occurred, time to occlusion is plotted below in Figure 3.3. In experiments where thrombus formation did not occur, time was recorded up to 1 hour after application of FeCl₃.

FeCl ₃ Conc	Clot Formation	Time to Occlusion (min:sec)
5%	No	-
10%	No	-
20%	No	-
20%	Yes	12:22
20%	Yes	10:40
20%	Yes	15:12
20%	Yes	17:02
20%	Yes	15:43
20%	Yes	16:55
20%	Yes	14:13
20%	Yes	14:41
20%	Yes	17:24
20%	Yes	10:31
20%	Yes	14:07
40%	Yes	2:30

Figure 3.3 - Time to Occlusion After Administration of 40% or 20% FeCl₃ to Rat Carotid Arteries.

Total vessel occlusion occurred in ~2.5min following administration of 40% FeCl₃ (n=1). Administration of 20% FeCl₃ (n=11) produced total vessel occlusion in a mean time of 14.44 ± 0.73 min.

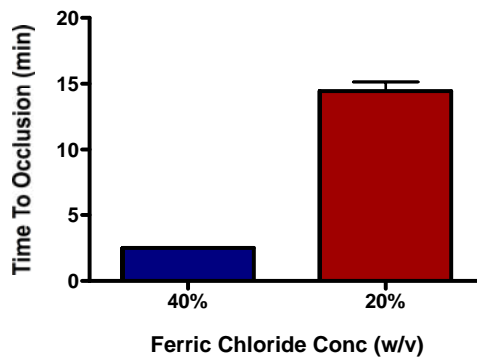


Figure 3.4 - Thrombus Formation in Carotid Arteries Following Treatment with FeCl₃.

Carotid artery sections from adult male Wistar rats stained with haematoxylin and eosin. a) Uninjured section of carotid artery. b) Carotid artery exposed to 5% FeCl₃ shows little sign of injury or thrombus formation. c) Carotid artery exposed to 20% FeCl₃ shows a dark ring around the inside of the vessel wall where FeCl₃ accumulates and destroys the endothelium, causing a fully occlusive thrombus to form within the vessel. d) Carotid artery exposed to 40% FeCl₃ shows a fully occlusive thrombus and extensive damage to the endothelium. Magnification x100.

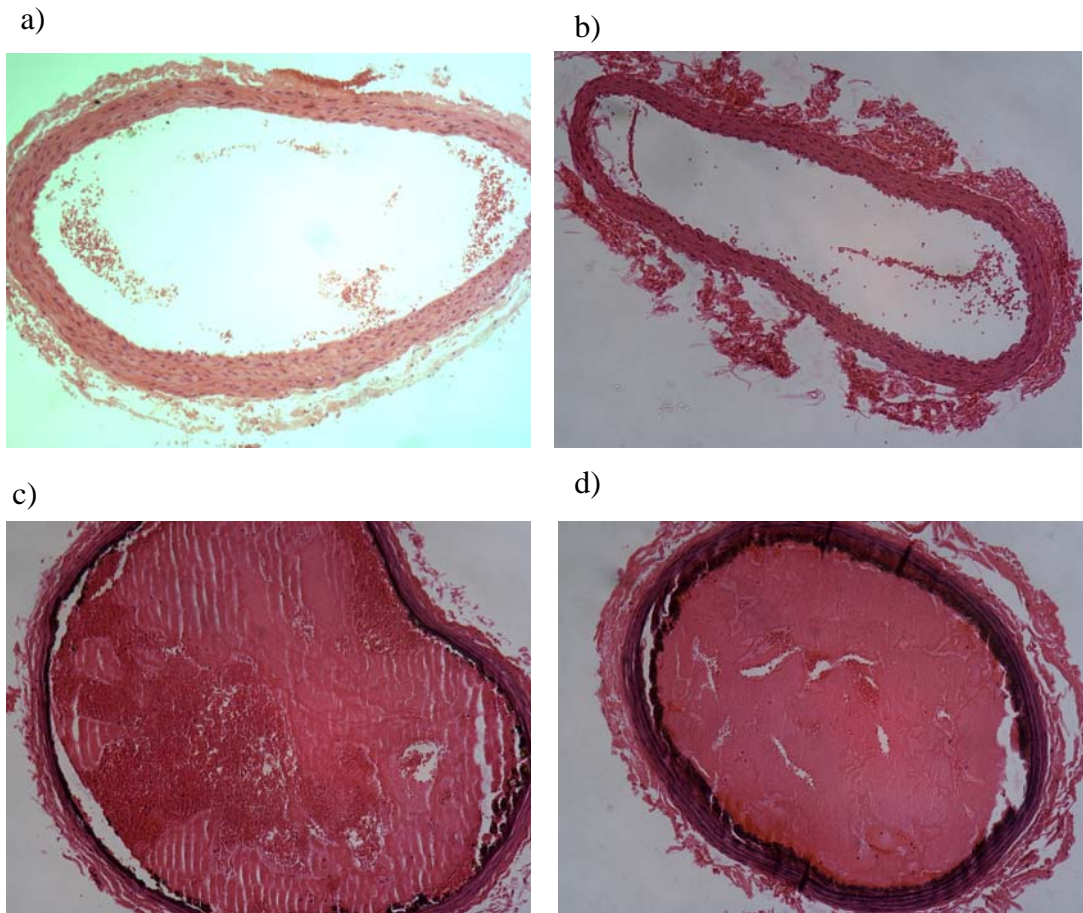
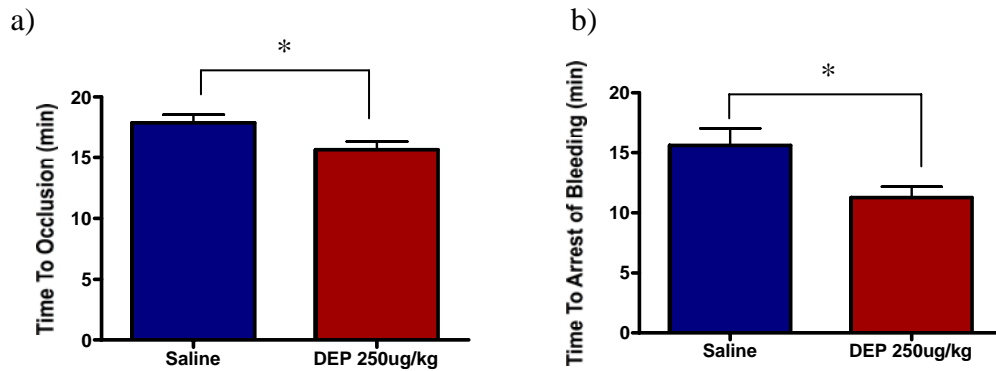


Figure 3.5 - Pilot Investigation of the Effect of DEP Administration on Thrombus Formation.

Intravenous administration of DEP (250 μ g/kg) to anaesthetised male Wistar rats 2 hours before application of FeCl₃ (20%) significantly reduced a) time to occlusion in response to administration and b) time to arrest of tail tip bleeding. All experiments were conducted 2 hours after DEP exposure. Columns represent Mean \pm SEM (n=6). Comparisons were made using Student's unpaired t-test. *P<0.05.



3.4 Discussion

The aims of the work described in this chapter were: (1) to develop suitable *in vivo* models of thrombosis; and (2) to perform a pilot study to determine whether these models were suitable for assessing the effects of diesel exhaust particulate (DEP) on thrombus formation. Initial investigations failed to produce reproducible, occlusive thrombosis using the Folts model in rats or mice. In contrast, application of 20% ferric chloride (FeCl_3) to the adventitial surface of the rat carotid artery consistently produced occlusive thrombus formation. Measurement of tail tip bleeding time provided a convenient and reproducible complementary measurement of clot formation in the same animals. Using these models, pilot investigations demonstrated that intravenous administration of DEP accelerated thrombosis; reducing both time to carotid artery occlusion and tail-tip bleeding time. These results indicate, therefore, that these methods are suitable for investigating the mechanisms underlying DEP-induced changes in endogenous thrombotic pathways.

Initial attempts to introduce an *in vivo* model of thrombosis focussed on the Folts model, as its combination of arterial stenosis and damage to the endothelium is considered a good representation of the processes contributing to coronary events in human atherothrombosis (Folts *et al.* 1982; Sturgeon *et al.* 2006). This model has been applied successfully to a variety of animals including: baboons (Wu *et al.* 2002), dogs (Folts *et al.* 1991; Hasa *et al.* 2001), pigs (Folts *et al.* 1991), rabbits (Dellamonica *et al.* 2008; Charbonneau *et al.* 2007; Sturgeon *et al.* 2006) and, more recently, to rats (Sturgeon *et al.* 2006) and mice (Sturgeon *et al.* 2006). Therefore, introduction of the technique to our labs was considered a realistic undertaking. The original intention was to induce thrombus formation in mice, as this would allow subsequent studies to use relevant transgenic animals (for example, PAI-1 knockout mice: Liu *et al.* 2009) and models of disease (such as the atherosclerotic apolipoprotein E deficient mouse; Schafer *et al.* 2003). However, although carotid artery blood flow measurements were successfully obtained in these animals, consistent reduction of blood flow by 50% proved very difficult (due to the small

size of the vessels). It may be for this reason that physical injury to the mouse carotid artery did not cause the generation of occlusive thrombus. Previous reports suggested that mice may be more resistant than rats or rabbits to induction of thrombosis using the Folts method (Sturgeon et al., 2006). Therefore, application of this technique was switched to the rat, in the hope that this would prove more reliable and reproducible and, given the size difference, technically less demanding. In rats it was possible to reduce blood flow by 50% through arterial stenosis, but when the artery was pinched to damage the endothelial layer the ligature had a tendency to loosen and allow normal blood flow to resume. Even in rats, formation of an occlusive thrombus was infrequent (occurring on only one occasion), and in this case the thrombus produced was so dense that it could not be fully embolised by agitating the artery. Communication with the Sturgeon group in Australia prompted the use of several methodological variations in an attempt to induce consistent thrombus formation. These included the use of Dysilk thread from South Australia as it was suggested that this would produce a more stable knot than standard suture silk. Despite these changes, no improvement in the development of thrombus was seen and further discussions with groups experienced with the Folts model led us to conclude that an alternative, more practical and reliable model of arterial thrombosis should be considered.

The application of FeCl_3 to the adventitial surface of the artery seemed an ideal alternative model. This approach is among the most common methods of inducing thrombus formation and has been applied to both large and small vessels (Denis *et al.* 1998; Farrehi *et al.* 1998). Following the earlier problems encountered applying the Folts model to mice, it was decided that rats would be used in these experiments. This model induces the formation of reactive oxygen species that cause local endothelial denudation, resulting in the formation of a platelet-rich thrombus, which is relevant to human atherosclerosis (Farrehi *et al.* 1998; Kurz *et al.* 1990; Denis & Wagner. 2007). Preliminary work showed that, as expected, exposure of the rat carotid artery to FeCl_3 produced occlusive thrombus formation that was concentration dependent and very reliable. Previous studies have used this model, with FeCl_3 concentrations ranging from 10-70% (Denis *et al.* 1998; Kurz *et al.* 1990;

Radomski *et al.* 2005; Wang & Xu. 2005), and so preliminary experiments were designed to find a suitable concentration; 20% FeCl₃ was used for subsequent experiments as it produced complete vessel occlusion in approximately 15 minutes with a high degree of consistency. Use of this concentration is not consistent with previous studies; two of which used mice as the experimental animal (Denis *et al.* 1998; Wang & Xu. 2005), which need a lower FeCl₃ concentration to produce occlusive thrombus formation than rats. The studies that used rats as the experimental animal used much higher FeCl₃ concentrations, such as 35% and 70% w/v (Kurz *et al.* 1990; Radomski *et al.* 2005). However the animals used in these studies were much older and larger (up to 450g) than the animals used in this thesis. In the study by Kurz *et al.* (1990), the application of 35% FeCl₃ appeared to cause total vessel occlusion in roughly the same time period with similar data consistency experienced with 20% FeCl₃ in this thesis. The result of 20% FeCl₃ application in this chapter showed that time to vessel occlusion was very consistent and reliable (coefficient of variation = 0.167). To summarise, the FeCl₃ model is a simple, inexpensive and reliable method of inducing thrombus formation. The only disadvantages to be considered are the so-called 'outside-inside' nature of the vascular injury, and the fact that the precise mechanism by which FeCl₃ triggers thrombosis is not well defined, or fully explained by simple endothelial denudation (Day *et al.* 2004). However, in this case the advantages of the model far outweigh the disadvantages.

The FeCl₃ technique is a model of platelet rich thrombus formation (as is the Folts model). It has been used previously to assess the effects of anti-platelet agents (Elg *et al.* 1999; Wang & Xu. 2004; Radomski *et al.* 2005) and bleeding disorders (Denis *et al.* 1998). Denis *et al.* (1998) showed that genetically-altered mice lacking von Willebrand factor rarely developed complete vessel occlusion following exposure to FeCl₃. These animals showed defects in platelet accumulation and significant impairment of platelet-vessel wall interaction, which lead to defective thrombus formation. The fibrinolytic system, particularly fibrinogen, also appears to play a role in this model. Ni *et al.* (2000) used fibrinogen-null mice to show that, while platelet deposition and onset of thrombus formation were the same as that of wild type mice,

the thrombi formed were unstable and generally embolised before complete vessel occlusion occurred. This suggests that both platelet function and the fibrinolytic system are essential for thrombus formation using this model, and therefore may provide a mechanism by which DEP accelerates thrombus formation. This mechanism requires further investigation, which will be discussed in the next chapter.

The tail tip method was also investigated in this chapter to be used alongside the FeCl₃ method. It is a straightforward technique that is easy to apply to animals undergoing the FeCl₃ technique. When used in these preliminary studies, this technique mirrored the results found following FeCl₃ application. However, previous studies investigating the effects of anti-coagulant and anti-platelet drugs have suggested that the tail tip technique shows different results than those found with the FeCl₃ technique (Elg *et al.* 1998; Wang & Xu. 2004). This suggests that the tail tip method may provide a different insight into the effects of DEP than the FeCl₃ method. This would help to differentiate the effects of DEP on haemostasis from the effects on platelet activation and aggregation, activation of the coagulation cascade and fibrin deposition.

Having established reliable, reproducible models of thrombus formation *in vivo*, it was important to determine whether they were able to detect the effects of DEP on this process. Consequently, a pilot study was performed based on previous work performed in a model of thrombus formation in the hamster (Nemmar *et al.* 2002; Nemmar *et al.* 2003). Initially, 500µg/kg DEP were administered intravenously to rats, based on the work of Nemmar and colleagues (Nemmar *et al.* 2002; Nemmar *et al.* 2003). A high DEP dose was chosen deliberately to ensure a maximal effect on thrombus formation. The finding that rats exposed to this dose only survived ~1 hour (before dying of apparent respiratory failure) was unexpected as the previous study had reported no such complications. Reduction of the DEP dose to 250µg/kg ensured that all rats survived the 2 hour experimental time point with no signs of respiratory distress. The method of administration, jugular vein injection, was also based on previous work (Nemmar *et al.* 2002). It appeared to be a suitable method, as the

experimental animal could be maintained under anaesthesia throughout the experiment, although using this method it would be very difficult to examine later timepoints in future experiments. Examinations of thrombogenesis 2 hours after administration of DEP was based on both previous studies (Vincent *et al.* 2001; Khandoga *et al.* 2004; Mills *et al.* 2005), and work from our own group, and should allow differences in thrombus formation to be detected without significant effects on inflammation. Furthermore, this short exposure also had practical benefits, as it would not have been desirable to maintain the animals under anaesthesia for a longer period.

The results obtained with these pilot experiments showed that intravenous administration of DEP significantly reduced time to vessel occlusion. This is consistent with previous work investigating the effects of injected polystyrene nanoparticles on thrombus formation (Nemmar *et al.* 2002). This route of administration has not been previously conducted with combustion-derived particles to investigate thrombus formation, although it has been used to show that DEP can cause systemic inflammation and both pulmonary and cardiac morphological alterations in rats at later time points (Nemmar *et al.* 2007). Carbon nanotubes (Bihari *et al.* 2009; Radomski *et al.* 2005), nanoparticulate titanium dioxide (Chen *et al.* 2009) and engineered quantum dots (Geys *et al.* 2008) have been shown to activate platelets and accelerate thrombus formation via intraperitoneal injection. Measurement of tail tip bleeding time produced results broadly consistent with those obtained from the application of FeCl₃ with a significant reduction in bleeding time following DEP injection. A similar reduction in bleeding time has been reported previously using the same method of DEP administration (Nemmar *et al.* 2007), which suggests that this is a useful additional technique in the investigation of the effects of DEP on thrombus formation.

Thus, in conclusion, the work reported in this chapter has led to the successful introduction of two reliable and reproducible models of thrombus formation *in vivo*. Both models were found to be sensitive to the administration of DEP, indicating that they were suitable for investigating the mechanisms responsible for enhanced

thrombogenesis following exposure to combustion derived diesel particles. This work, therefore, paved the way for more detailed mechanistic investigations, detailed in chapter 4, aimed at determining the importance of direct interaction with the vascular wall and (systemic and pulmonary) inflammation in mediating the pro-thrombotic effects of DEP.

Chapter 4

Effects of Diesel Exhaust Particulate on Thrombus Formation In Vivo

4.1 Introduction

Method development and pilot investigations (Chapter 3) confirmed the ferric chloride (FeCl_3) and tail tip models could be used to successfully detect changes in thrombus formation following exposure to diesel exhaust particulate (DEP). The work described in this chapter was designed to clarify the mechanisms through which DEP enhance thrombus formation.

Previous studies have also shown that DEP have an effect on increasing thrombus formation, by affecting expression of factors that enhance, or inhibit the breakdown of thrombi; this has been shown in both pre-clinical and clinical studies (Nemmar *et al.* 2002; Nemmar *et al.* 2003; Mills *et al.* 2007; Lucking *et al.* 2008). The exact mechanism behind this effect, however, remains unclear. The effects of DEP may be due to direct interaction with the cardiovascular system, following translocation from the lungs (Nemmar *et al.* 2004), or secondary to pulmonary (Ito *et al.* 2000; Nemmar *et al.* 2003; Singh *et al.* 2004; Ahn *et al.* 2008; Yokota *et al.* 2008) and/or systemic (Salvi *et al.* 1999; Yokota *et al.* 2005; Tornqvist *et al.* 2007; Mills *et al.* 2008; Nemmar *et al.* 2008; Nemmar *et al.* 2009) inflammation caused by a spill-over of inflammatory mediators from the lung into the circulation. The pathways via which DEP has already been shown to cause changes in thrombus formation are alterations in platelet activity (Lucking *et al.* 2008) and impairment of the endogenous fibrinolytic system (Mills *et al.* 2005; Mills *et al.* 2007), although changes in fibrinogen and coagulation factors are also possible.

In order to address these questions it was necessary to administer DEP both via the lungs and directly into the bloodstream. Administration to the lungs is the most relevant exposure route to air pollution experienced by humans. In practice pulmonary administration can be achieved either by inhalation of a DEP aerosol or by intrapulmonary instillation of a particulate suspension. Superficially, inhalation appears to be the best method to adopt and, indeed, it has been used many times in the study of DEP on the cardiovascular system (Vallyathan *et al.* 1986; Lewis *et al.* 1986; Vincent *et al.* 2001; Campen *et al.* 2003; Furuyama *et al.* 2006; Wold *et al.*

2006; Simkhovich *et al.* 2007; Cherng *et al.* 2009). There is some concern, however, that this may be problematic in rodents. A previous study using rats indicated that only a small percentage of inhaled DEP actually reached the lungs (Chan *et al.* 1981; Duffin *et al.* Unpublished observations). This is most likely due to the complex nasal structure in these animals, which can filter out a large component of the particulate before it reaches the lungs. Animals exposed to whole cage exposure to aerosols can also experience particle deposition on their coats, or ingestion, rather than inhalation, of particles, making the exact exposure difficult to calculate. In contrast, intrapulmonary instillation has the advantage of ensuring administration of a well-defined particulate dose directly into the lungs (Nemmar *et al.* 2002; Nemmar *et al.* 2003; Nemmar *et al.* 2004; McQueen *et al.* 2007), as well as allowing delivery of different particle types that are practically difficult to deliver by inhalation. By comparison with intrapulmonary exposure, direct administration of particulate solutions to the bloodstream is relatively straightforward. Particle suspensions have been administered directly by intravenous injection in a number of previous animal studies (Nemmar *et al.* 2002a; Radomski *et al.* 2005; Geys *et al.* 2008; Nemmar *et al.* 2009). These studies show that injection of nanoparticles can cause systemic and pulmonary inflammation, increased thrombogenicity via platelet activation and possible involvement of the coagulation cascade. However, only the study by Nemmar *et al.* (2009) investigated the effects of DEP, and in this study spontaneously hypertensive rats were used instead of healthy animals. This study will significantly enhance the field by investigating multiple effects of DEP, and control particles, on healthy animals. Comparing the responses to intravenous and intratracheal administration should also clarify the contributions of direct interaction with the cardiovascular system and pulmonary activation to DEP-mediated increases in thrombus formation.

4.1.1 Hypothesis

Exposure of rats to diesel exhaust particulate (DEP) will increase thrombogenesis, independent of pulmonary or systemic inflammation, possibly through changes in platelet function and/or the endogenous fibrinolytic system.

4.1.2 Aims

The aims of the work described in this chapter were to determine whether:

- i. exposure to DEP increases thrombus formation in complementary experimental models.
- ii. the ability of DEP to enhance thrombogenicity is influenced by the route of exposure.
- iii. enhanced thrombogenesis in response to DEP administration is secondary to systemic or pulmonary inflammation.
- iv. enhanced thrombogenesis in response to DEP administration is due to changes in platelet activity or in the endogenous fibrinolytic system.
- v. the effects of DEP on inflammation and thrombogenicity are mirrored by the use of other particles (CB and DQ12 quartz), or if these affects are DEP specific.

4.2 Methods

4.2.1 Animals

Adult male Wistar rats (100-125g) were obtained from Charles River Laboratories (Wilmington, MA, USA). All animals were housed for at least one week, with constant access to food and water and a 12 hour light/dark cycle, before use in the following experiments.

4.2.2 Preparation and Administration of Particulate Suspensions

4.2.2.1 Preparation of Particulate Suspensions

DEP and carbon black (CB) solutions (1mg/ml) and quartz (DQ12) solution (0.25mg/ml) were prepared in sterile 0.9% saline solution by sonication (Status Homogenisers; Probe from Philip Harris Scientific, US70; Lichfield, UK) at 70% power for 5 minutes.

4.2.2.2 Pulmonary Instillation of Particulates

Intratracheal instillation was performed by Dr Rodger Duffin, as described previously (McQueen *et al.* 2007). Rats were anaesthetised briefly using isoflurane (Merial, Essex, UK) and an equivalent volume of DEP (0.5ml of a 1mg/ml suspension) or 0.9% sterile saline (0.5ml) was applied directly into the trachea. Animals were allowed to recover from the anaesthetic and housed for 2, 6 or 24 hours before being assessed for thrombus formation and markers of inflammation. This range of timepoints allows examination of immediate and longer-lasting effects. CB (0.5ml of a 1mg/ml solution) was administered to examine the results of clean carbon nanoparticles in order to determine the DEP specific effects that may be a result of the volatiles, metals and organic compounds associated with the surface of DEP. DQ12 solutions (0.5ml of a 0.25mg/ml solution) were administered to examine

the results of large non-carbon particles that cannot translocate into the systemic circulation. These control particles were examined 6 hours after administration.

4.2.2.3 Intravenous Administration of Particulates

Intravenous administration of experimental solutions and saline was achieved by tail vein injection in rats briefly anaesthetised with inhaled isoflurane. Rats were weighed and received either DEP (0.5mg/kg in 0.2ml saline) or sterile saline (0.2ml). This concentration was chosen based on previous injection experiments in mice (Yoshino *et al.* 1999). Animals were allowed to recover from the procedure for 2, 6 or 24 hours before being anaesthetised and assessed for thrombus formation and markers of inflammation. CB (0.5mg/kg in 0.2ml saline) was administered and the effects examined 2 hours after tail vein injection. DQ12 was not examined in the tail vein injection experiments, as this large particle would not be able to translocate from the lungs into the bloodstream.

4.2.3 Assessment of Clotting Time

4.2.3.1 Ferric Chloride-Mediated Induction of Thrombosis

Induction of arterial thrombus by direct application of ferric chloride was performed as described (Chapter 2.3.3.1). Briefly, 20% FeCl₃ was applied topically to the carotid artery for 10 minutes before being carefully removed. Blood flow through the artery was recorded, with timing started from the application of FeCl₃ and stopped when blood flow reached 0ml/min.

4.2.3.2 Time to Arrest of Bleeding

In all animals, the time to arrest of tail tip bleeding (Tanaka *et al.* 1998) was assessed by removing the tail tip (5mm) with a scalpel and cleaning the wound every 10 seconds until no blood was transferred from the tail to the swab (Chapter 2.3.3.2).

4.2.3 Impact of Particulate Administration on Pulmonary Inflammation

4.2.3.1 Collection and Processing of Bronchoalveolar Lavage Fluid

After rats had been killed by exanguination, the lungs were cannulated and lavaged with 1 × 8ml of sterile saline. This first lavage was retained for cellular analysis of the bronchoalveolar lavage (BAL) fluid profile. The lungs were then lavaged a further 3 times with 8ml sterile saline and the BAL fluid from these lavages was combined. All samples were centrifuged at 180g for 5 mins at 4°C. The supernatant from the first lavage was separated into 1ml aliquots and stored at -80°C for further analysis. The supernatant from the 2nd-4th lavages was removed and discarded, and the cell pellet was resuspended in 1ml sterile saline and added to the cell pellet from the first lavage.

4.2.3.2 Pulmonary Cell Count Following Particulate Administration

The resuspended cell pellets were analysed using an automatic cell counter (ChemoMetec A/S. Denmark). Briefly, 50µl of the cell suspension was added to 50µl lysis reagent A-100 and 50µl stabilizing reagent B (ChemoMetec A/S. Denmark). The solution was then loaded into a Nucleocassette (ChemoMetec A/S. Denmark) for the cell count. Where the initial cell count was too high to give an accurate reading, the cell suspension was diluted 1 in 10 using sterile saline and analysed again. The number of cells counted using the automatic cell counter was then multiplied by the

dilution factor to give the total number of cells recovered from the bronchoalveolar lavage.

4.2.3.3 Differential Cell Counts Following Particulate Administration

Cytocentrifuge smears were prepared by adding 1×10^6 cells from the previous cell suspension to 300µl saline + 0.1% bovine serum albumen (BSA). Samples were centrifuged (300g, 3 mins, room temperature) onto a microscope slide (Superfrost Plus, 75mm x 25mm. VWR International Ltd. Leicestershire, UK), and stained with Diff-Quick (Raymond A Lamb. London, UK). 300 cells per slide were counted at random and the results expressed as total numbers of each cell type in the lung lavage by referring back to the original cell count.

4.2.3.4 Effects of Particulates on Pulmonary Cytotoxicity

The cytotoxic effects of DEP, CB and DQ12 on cytotoxicity and pulmonary exudate were assessed using a cytotoxicity detection kit (LDH. Roche Diagnostics Ltd, Burgess Hill, UK) on BAL fluid samples. Briefly, 100µl of BAL fluid sample was added to 100µl working solution in a 96-well plate. Positive control wells also received 5µl lysis solution, while negative control well received deionised water instead of the cell suspension. The plate was read at 490nm with a 630nm reference filter, and LDH levels were calculated as a percentage value of the difference between the maximum and minimum control.

4.2.3.5 Effects of Particulates on Pulmonary Protein Levels

Total protein content in BAL fluid was measured using a bicinchoninic acid (BCA) protein assay (Thermo Scientific. Northumberland, UK). Briefly, 10µl of sample BAL fluid and BSA standards from 0.025 to 2mg/ml were added to a 96-well plate. A 1:50 solution of copper (II) sulphate and bicinchoninic acid was made and 190µl

of the working solution was added to each well. The plate was incubated (37°C, 30 mins), then read using a plate reader at 570nm. The known standard concentrations (0.125-2mg/ml) were used to calculate sample protein concentrations.

4.2.4 Impact of Particulate Administration on Platelet-Monocyte Interactions

4.2.4.1 Collection of Blood and Plasma

Blood was collected from the abdominal aorta and mixed (10:1) with 3.8% sodium citrate solution. Fresh whole blood was used immediately for flow cytometry experiments. Whole blood was centrifuged at 1500g for 5 mins to obtain plasma, which was separated into 0.5ml aliquots and stored at -80°C until further analysis.

4.2.4.2 Flow Cytometry

In order to characterise platelet-monocyte and platelet-platelet aggregation following exposure to particulates, flow cytometric analysis of whole blood was carried out to measure the percentage of cells positive for platelet and monocyte cell surface markers. Fluorescent-conjugated antibodies (BD Biosciences; Oxford, UK) can be used to detect specific cell surface markers; fluorescein isothiocyanate (FITC)-labelled anti-rat CD42d antibody was used to label platelets, phycoerythrin (PE)-labeled hamster anti-mouse CD61 was used to label monocytes and PE-labeled hamster IgG1 κ was used as a negative isotype control. Whole blood (30 μ l) was added to 30 μ l of diluted antibody (CD42d - 1/100 dilution in PBS, CD61 and isotype control 1/50 dilution in PBS. Dilutions were based on antibody concentrations and preliminary experiments), or 15 μ l of each antibody when double labelling was performed. Samples were incubated at room temperature in the dark for 20 minutes before the addition of 500 μ l 1x FACS lysing solution (BD Biosciences; Oxford, UK). They were then incubated for a further 10 minutes before being analysed via

flow cytometry (BD FACScan flow cytometer using CellQuest software and FlowJo software for analysis).

4.2.5 Analysis of Inflammatory Markers by ELISA

In order to determine whether administration of particulate solutions caused systemic or pulmonary inflammation, plasma (collected as described in Chapter 4.2.4.1) and BAL (collected as described in Chapter 4.2.3.1) fluid samples were assessed for levels of interleukin 6 (IL-6. DuoSet ELISA Rat IL-6 kit), C reactive protein (CRP. DuoSet ELISA Rat CRP kit) and tumour necrosis factor alpha (TNF α . DuoSet ELISA Rat TNF α kit). Due to problems with these assays in rat plasma, both DuoSet and Quantikine ELISA kits were examined for IL-6 and TNF α levels in plasma (all kits from R&D Systems. Abingdon, UK). However, these kits were also unsuccessful; only CRP could be reliably measured in plasma (See Results and Chapter 2.5 and 2.6 for full details).

4.2.6 Analysis of the Thrombolytic System by ELISA

In order to determine whether administration of particulate solutions altered endogenous fibrinolysis, plasma samples were assessed for levels of tissue plasminogen activator (t-PA), plasminogen activator inhibitor (PAI-1), fibrinogen and D dimer. Rat t-PA antigen, rat PAI-1 antigen and rat fibrinogen antigen ELISAs were obtained from Patricell Ltd. (Nottingham, UK. See Chapter 2.5.2). D dimer ELISAs were performed by Pamela Dawson (Haematology department; Royal Infirmary of Edinburgh).

4.2.7 Histological Assessment of Thrombus and Particle Translocation

Following surgery, both carotid arteries, a section of aorta, one whole kidney and a section of liver were removed and fixed in neutral buffered formalin for 24 hours

before being processed and embedded in paraffin wax for histological analysis. Serial sections (4µm) were cut using a microtome and the sections were stained using the haemotoxilin and eosin method. Samples were then cover slipped and photographed at 1000x magnification, and compared with lung sections taken 2 hours after DEP instillation for visualisation of DEP.

4.2.8 Statistics

Data are expressed as mean \pm SEM. Results were analysed using GraphPad Prism software. Comparison of 2 groups was performed using an unpaired two-tailed t-test. Comparison of 3 or more groups was performed using a one-way ANOVA followed by a Tukey's multiple comparison post-test. Comparison of 2 groups at 3 different time-points was achieved using a two-way ANOVA with a Bonferroni post-test. Significance was assumed when $P < 0.05$.

4.3 Results

4.3.1 DEP Administration Accelerates Thrombosis

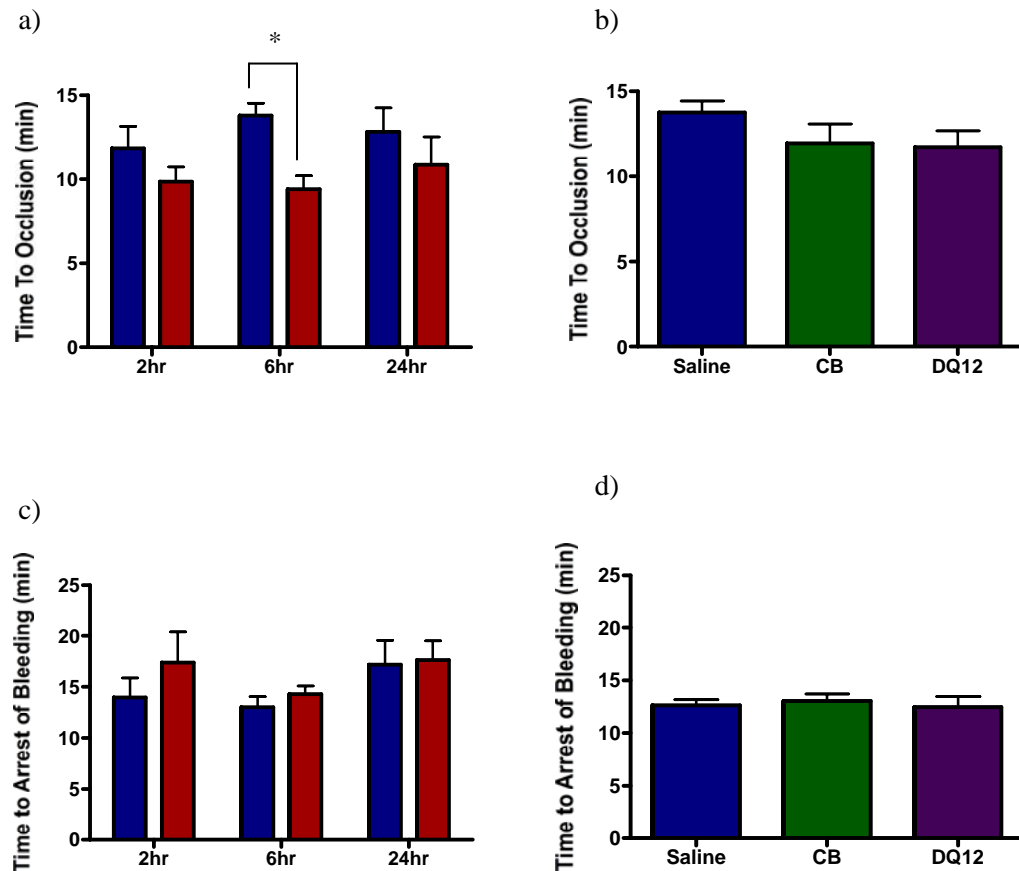
4.3.1.1 Effects of Particulate Instillation on Thrombus Formation

In rats exposed to intratracheal instillation of vehicle (saline), the time to complete thrombotic occlusion of the carotid artery (Figure 4.1) was slightly faster (~13mins) than vehicle-treated (~17mins) rats tested during the pilot investigations (Chapter 3.3.3). However, the response of vehicle-treated animals appeared to be relatively consistent at the different timepoints examined in this study. Instillation of DEP produced an apparent reduction in the time to occlusion 2, 6 and 24 hours after administration (Figure 4.1a). This effect only achieved significance, however, 6 hours after administration ($P < 0.05$). Both CB and DQ12 also produce an apparent reduction in time to occlusion 6 hours after administration, but this did not achieve statistical significance (Figure 4.1c).

Time to arrest of tail tip bleeding (~13min) in vehicle-treated rats (Figure 4.1b & 4.1d) was also slightly faster than that observed for controls (~15min) during the model development (Chapter 3.3.3), with the exception of the vehicle-treated group 24 hours after instillation (~16min). In contrast to the changes seen in carotid artery thrombosis, intratracheal instillation of DEP had no effect on time to arrest of bleeding at any of the time-points studied (Figure 4.1b). Similarly, exposure to CB or DQ12 did not alter time to arrest of bleeding when studied 6 hours after administration (Figure 4.1d).

Figure 4.1 - Effects of Particle Instillation on Thrombus Formation *In Vivo*.

a) Time to carotid artery occlusion following ferric chloride (FeCl_3) application 2, 6 and 24 hours after instillation of saline (Blue columns) or diesel exhaust particulate (DEP - 0.5mg; Red columns). Results suggest a reduction in time to occlusion following DEP instillation at all 3 timepoints, however, significance was only achieved at the 6hr timepoint ($P < 0.05$). b) Time to carotid artery occlusion following FeCl_3 application 6 hours after instillation of saline, carbon black (CB - 0.5mg; Green columns) or quartz (DQ12 - 0.125mg; Purple columns). c) Time to arrest of bleeding following tail tip removal. Results show no significant differences between saline and DEP (0.5mg) at any of the 3 timepoints. d) Time to arrest of bleeding following tail tip removal. Results show no significant differences between saline, CB (0.5mg) and DQ12 (0.125mg) at the 6 hour timepoint. Columns represent mean \pm SEM ($n=6$ for all groups). * $P > 0.05$.



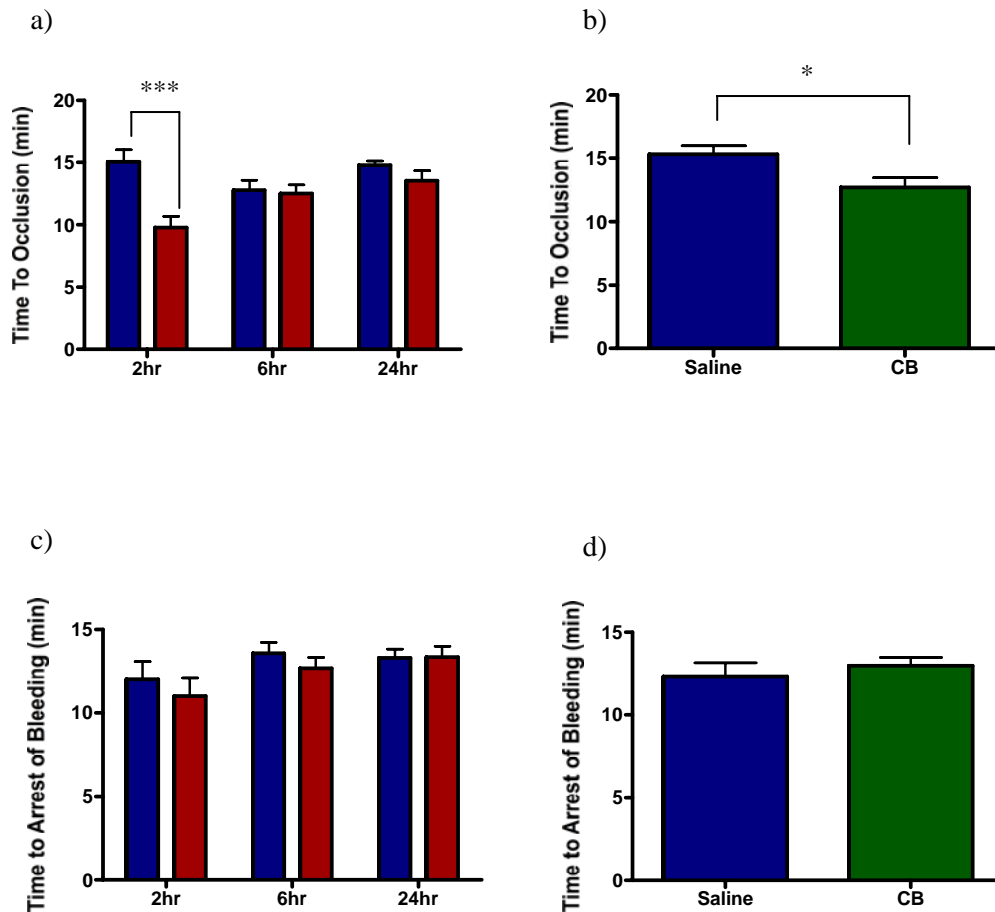
4.3.1.2 Effects of Particulate Tail Vein Injection on Thrombus Formation

In rats exposed to intravenous injection of vehicle (saline), the time to complete thrombotic occlusion of the carotid artery (Figure 4.1) was slightly faster (~14mins) than vehicle-treated (~17mins) rats tested during the pilot investigations (Chapter 3.3.3). However, the response of vehicle-treated animals appeared to be relatively consistent at the different timepoints examined in this study. Intravenous injection of DEP significantly reduced time to occlusion 2 hours after administration (Figure 4.2a). This effect was not observed 6 and 24 hours after administration. CB also significantly reduced time to occlusion 2 hours after administration when compared with saline ($P=0.0293$; Figure 4.2c).

Time to arrest of tail tip bleeding (~13min) in vehicle-treated rats (Figure 4.2b & 4.1d) was also slightly faster than that observed for controls (~15min) during the model development (Chapter 3.3.3). In contrast to the changes seen in carotid artery thrombosis, intravenous injection of DEP had no effect on time to arrest of bleeding at any of the time-points studied (Figure 4.1b). Similarly, exposure to CB 2 hours after injection did not alter time to arrest of bleeding (Figure 4.2d).

Figure 4.2 - Effects of Particle Injection on Thrombus Formation *In Vivo*.

a) Time to carotid artery occlusion 2, 6 and 24hrs following ferric chloride (FeCl_3) application after tail vein injection of saline (Blue columns) or diesel exhaust particulate (DEP - 0.5mg/kg: Red columns). Results show a significant reduction in time to occlusion following DEP injection at the 2 hour timepoint ($P < 0.001$). b) Time to carotid artery occlusion following FeCl_3 application 2 hours after tail vein injection of saline or carbon black (CB - 0.5mg/kg: Green columns). Results show a significant reduction in time to occlusion following CB injection ($P = 0.0293$). c) Time to arrest of bleeding following tail tip removal. Results show no significant differences between saline and DEP at any of the 3 timepoints. d) Time to arrest of bleeding following tail tip removal. Results show no significant differences between saline and CB at the 2 hour timepoint. Columns represent mean \pm SEM ($n = 6$ for all groups). * $P > 0.05$, *** $P > 0.001$.



4.3.2 Effects of Particulate Administration on Pulmonary Inflammatory Cell Count

4.3.2.1 Effects of Particulate Instillation on Pulmonary Cell Count and Cell Differential

Cytospins of cells taken from BAL fluid show the infiltration of neutrophils following instillation of DEP, CB and DQ12 (Figure 4.3). These pictures also show DEP and CB particle accumulation in, or on the surface of, macrophages.

Instillation of DEP ($P < 0.001$; Figure 4.4a) or either control particle (CB; $P < 0.05$ or DQ12; $P < 0.05$; Figure 4.4b) produced a significant increase, compared with saline-treated controls, in the total number of cells counted in BAL fluid 6 hours after intra-tracheal administration. In contrast, there were no increases (compared with control) in cell count in BAL samples 2 or 24 hours after administration of DEP. Differential analysis indicated that the particle-induced increase in cell numbers 6 hours after administration was due to an increase ($P < 0.001$) in the number of neutrophils (Figure 4.4d), rather than macrophages ($P > 0.05$; Figure 4.4c) or other cell types (data not shown). The percentage of pulmonary cells identified as neutrophils rose from 6.2% in animals instilled with saline, to 67.5% due to DEP, 6 hours after instillation. Intriguingly, the number of neutrophils remained slightly elevated ($P < 0.05$) 24 hours after treatment with DEP, despite the total cell count decreasing to levels similar to controls. The increased cell count in CB- and quartz-treated rats was also produced largely by an increase in the number of neutrophils in each sample (Figure 4.4e). Again there were no significant changes in the number of macrophages (Figure 4.4e) or other cell types (data not shown) in this experiment. The percentage of pulmonary cells identified as neutrophils rose from 6.0% in animals instilled with saline, to 75.3% due to CB and 77.9% due to DQ12, 2 hours after instillation.

Figure 4.3 - Cytospins Showing Pulmonary Inflammatory Cell Infiltration Following Particle Instillation.

Cells recovered from bronchoalveolar lavage samples and stained using haematoxylin and eosin. a) 2 hour instillation with saline shows macrophages only (large circular cells, single-lobed nucleus. Identified by arrow). b) 2 hour instillation with 0.5mg diesel exhaust particulate (DEP) shows mostly macrophages with some neutrophil infiltration (smaller cells, multi-lobed nucleus. Identified by arrow). DEP uptake by, or adhesion to, macrophages can also be seen. c) 6 hours after instillation with saline shows macrophages only. d) 6 hour instillation with 0.5mg DEP shows extensive neutrophil infiltration. DEP uptake by macrophages can also be seen. e) 6 hours after instillation with 0.5mg carbon black (CB) shows extensive neutrophil infiltration. CB uptake by macrophages can also be seen. f) 6 hour instillation with 0.125mg quartz (DQ12) shows extensive neutrophil infiltration. DQ12 particles are not visible. g) 24 hour instillation with saline shows macrophages only. h) 24 hour instillation with 0.5mg DEP shows mostly macrophages with some neutrophil infiltration. DEP uptake by macrophages can also be seen. Magnification x400. Pictures taken are of cells at the same concentrations (1×10^6 cells/slide); apparent changes in cell number/density are an artefact of the cytopsin process.

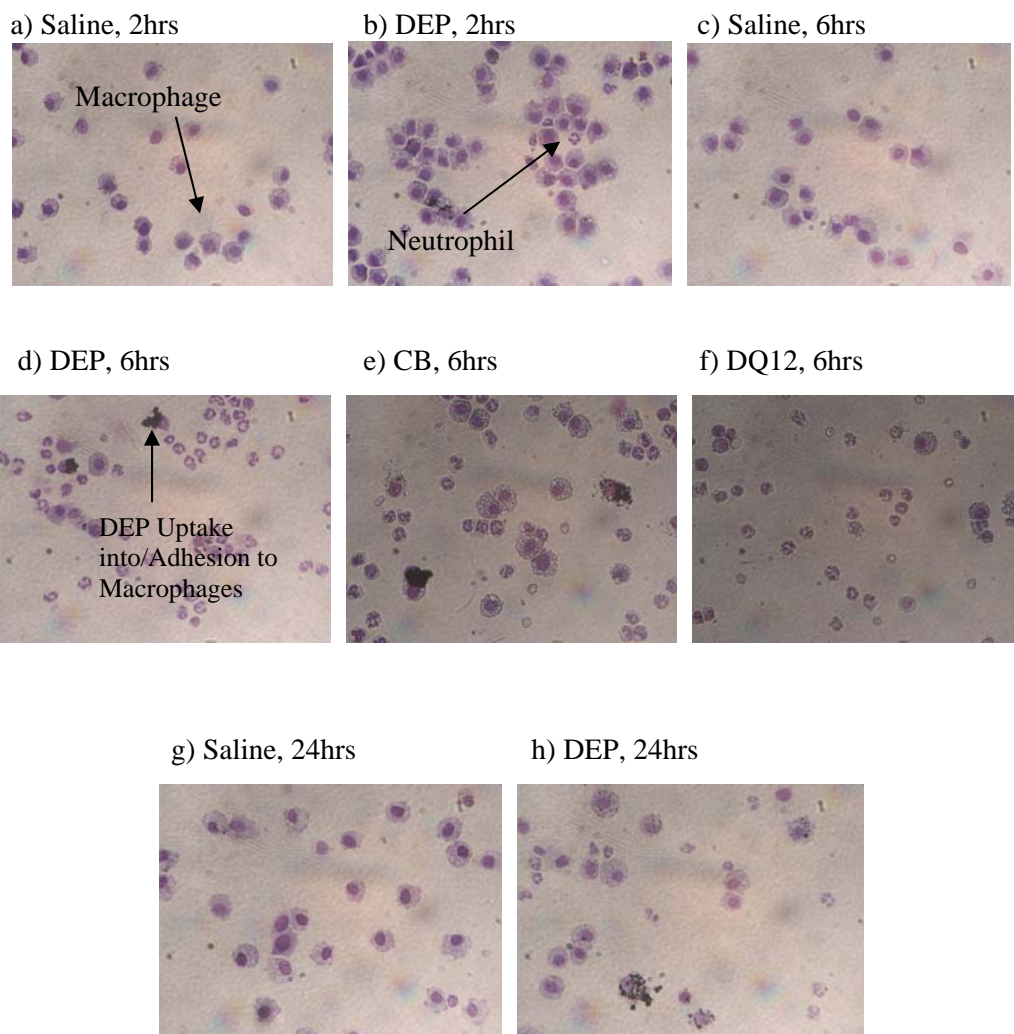
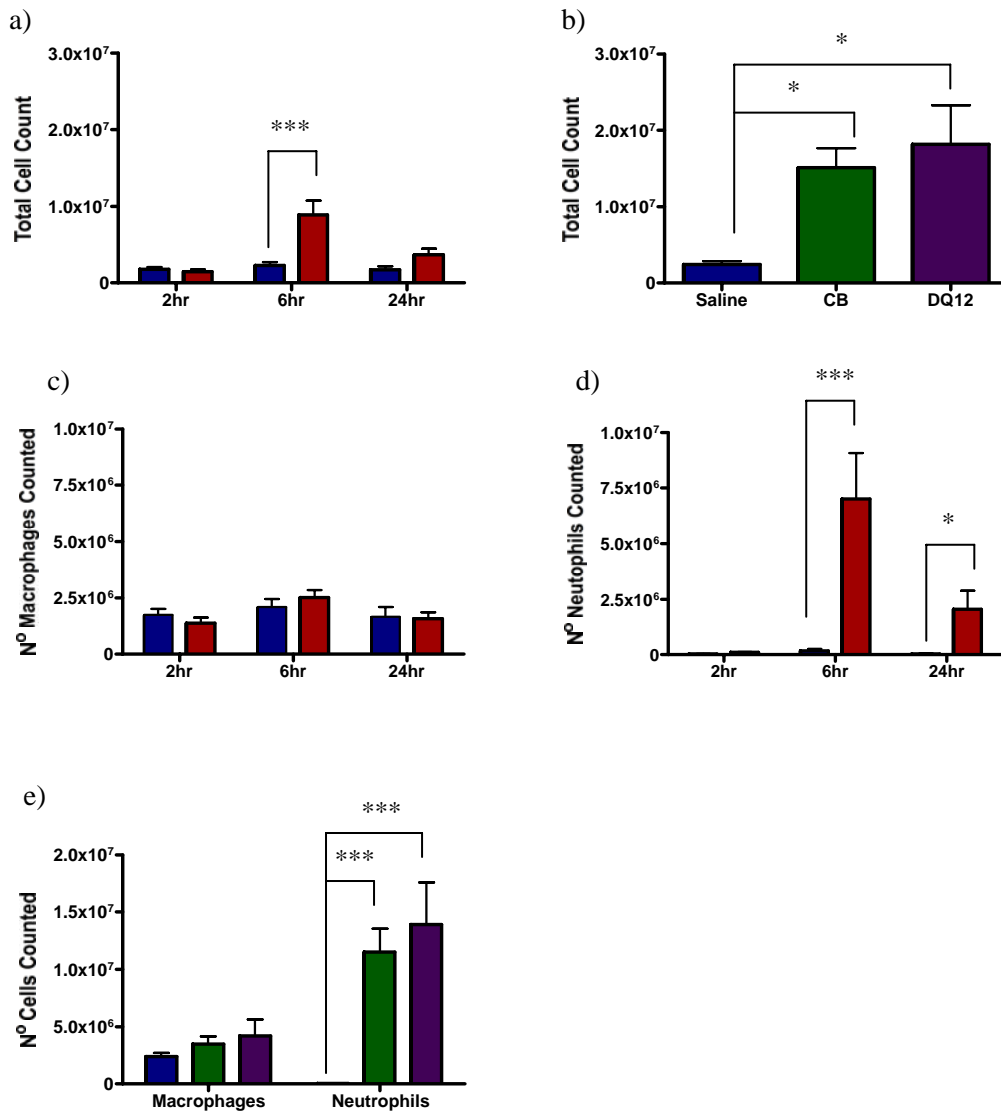


Figure 4.4 - Pulmonary Cell Count and Cell Differentials Following Particle Instillation *In Vivo*.

a) Total cell count of BAL samples 2, 6 and 24 hours after instillation of saline (Blue columns) or diesel exhaust particulate (DEP - 0.5mg; Red columns). Results show a significant increase in the number of cells counted 6 hours after DEP instillation compared with saline ($P<0.001$). b) Total cell count results show a significant increase in the number of cells counted 6 hours after carbon black (CB - 0.5mg; Green columns. $P<0.05$) or quartz (DQ12 - 0.125mg; Purple columns $P<0.05$) instillation when compared with saline. c) Cell differential analysis shows no significant changes in the number of macrophages recovered from BAL samples 2, 6 and 24 hours after instillation of saline or DEP. d) Cell differential shows significant increases in the number of neutrophils recovered following instillation of DEP when compared with saline at the 6 hour ($P<0.001$) and 24 hour time-points ($P<0.05$). e) Cell differential analysis shows significant differences in the number of macrophages or other cells between any of the 3 groups. However there was a significant increase in the number of neutrophils recovered following both CB ($P<0.001$) and DQ12 ($P<0.001$) instillation when compared with saline. Columns represent mean \pm SEM ($n=6$ for all groups). * $P>0.05$, *** $P>0.001$.



4.3.2.1 Effects of Particle Tail Vein Injection on Pulmonary Cell Count and Cell Differential

Cytospins of cells taken from BAL fluid show no neutrophil infiltration following injection of DEP or CB (Figure 4.5). These pictures show no particle accumulation in, or on the surface of, macrophages.

The total number of cells collected from BAL fluid of injected mice was markedly lower than for instilled mice ($\sim 4\text{-}5 \times 10^6$ vs. $\sim 1\text{-}2 \times 10^7$). Tail vein injection of DEP did not increase the number of cells in BAL fluid samples at any of the time points (2, 6 or 24) hours assessed (Figure 4.6a). Similarly, injection of CB did not alter total cell number in BAL samples 2 hours following administration (Figure 4.6b). Cell differential analysis confirmed that the cells in all BAL samples from all injected rats were almost exclusively macrophages (Figures 4.6c & 4.6d).

Figure 4.5 - Cytospins Showing Pulmonary Inflammatory Cell Populations Following Particle Injection.

Cells recovered from bronchoalveolar lavage samples stained using haematoxylin and eosin. a) 2 hours after injection with saline. b) 2 hours after injection with 0.5mg/kg DEP c) 6 hours after injection with 0.5mg/kg CB. d) 6 hours after injection with saline. e) 6 hours after instillation with 0.5mg/kg DEP. f) 24 hours after injection with saline. g) 24 hours after instillation with 0.5mg/kg DEP. All lavages show only macrophages, no neutrophil infiltration was observed following any of the injections and there was no evidence of particulates in the samples. Magnification x400.

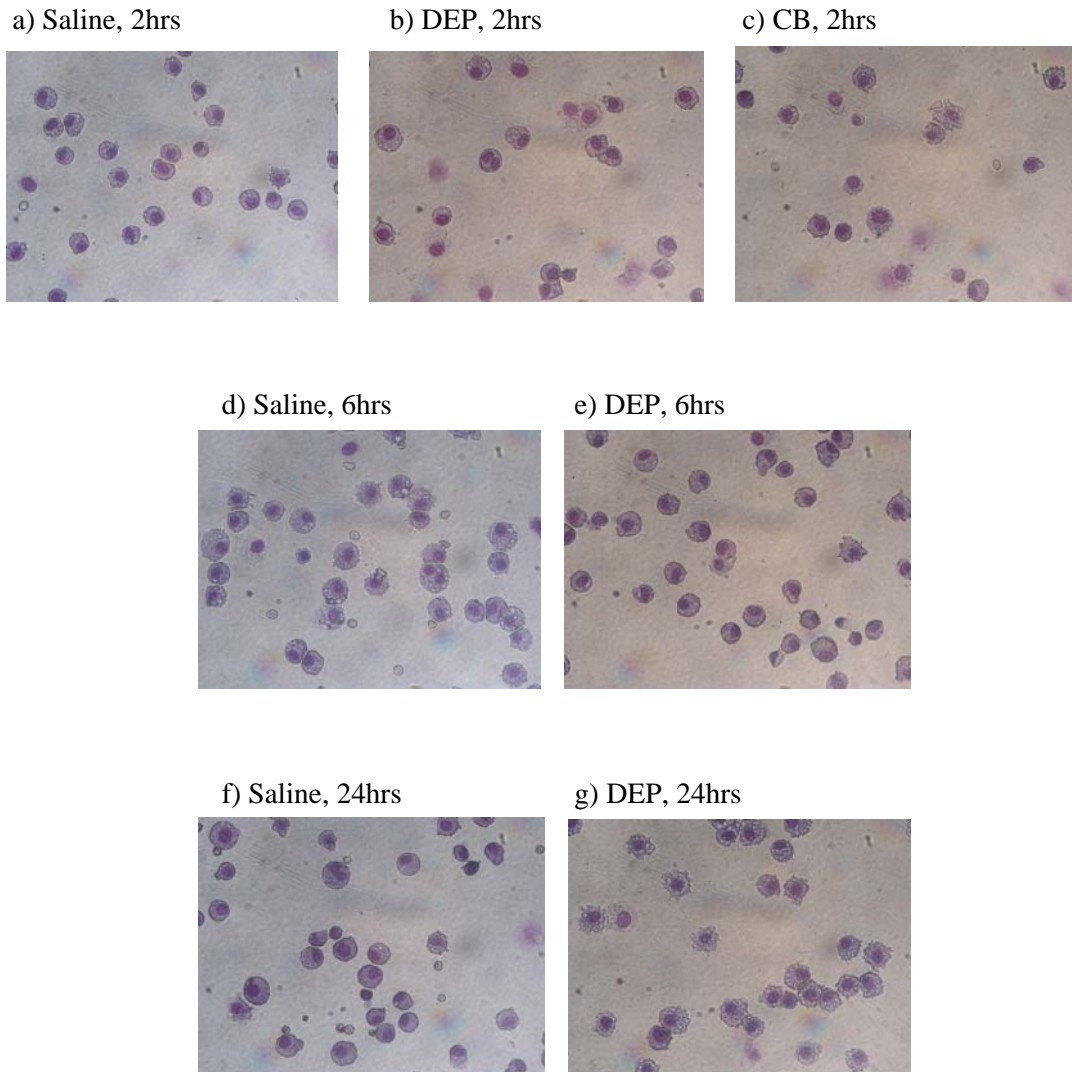
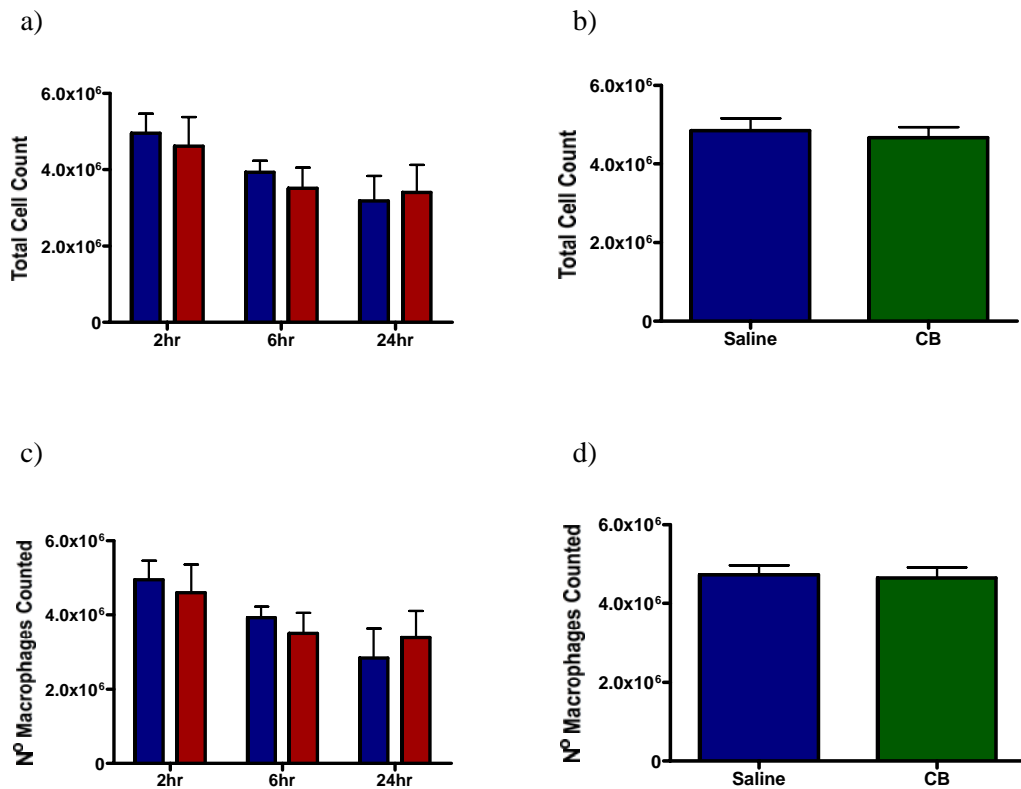


Figure 4.6 - Pulmonary Cell Count and Cell Differentials Following Particle Injection *In Vivo*.

a) Cell count following BAL 2, 6 and 24 hours after tail vein injection of saline (Blue columns) and diesel exhaust particulate (DEP - 0.5mg/kg; Red columns). b) Cell count following BAL 2 hours after tail vein injection of saline and carbon black (CB - 0.5mg/kg; Green columns). c) Cell differential shows the number of macrophages recovered following BAL 2, 6 and 24 hours after instillation of saline and DEP. e) Cell differential for cells recovered during BAL 2 hours after tail vein injection of saline and CB. Results show no significant differences between DEP or CB and saline at any of the timepoints tested and no significant neutrophil infiltration was observed under any condition (data not shown). Columns represent mean \pm SEM (n=6 for all groups).



4.3.3 Effects of Particulate Administration on Pulmonary Cell Viability

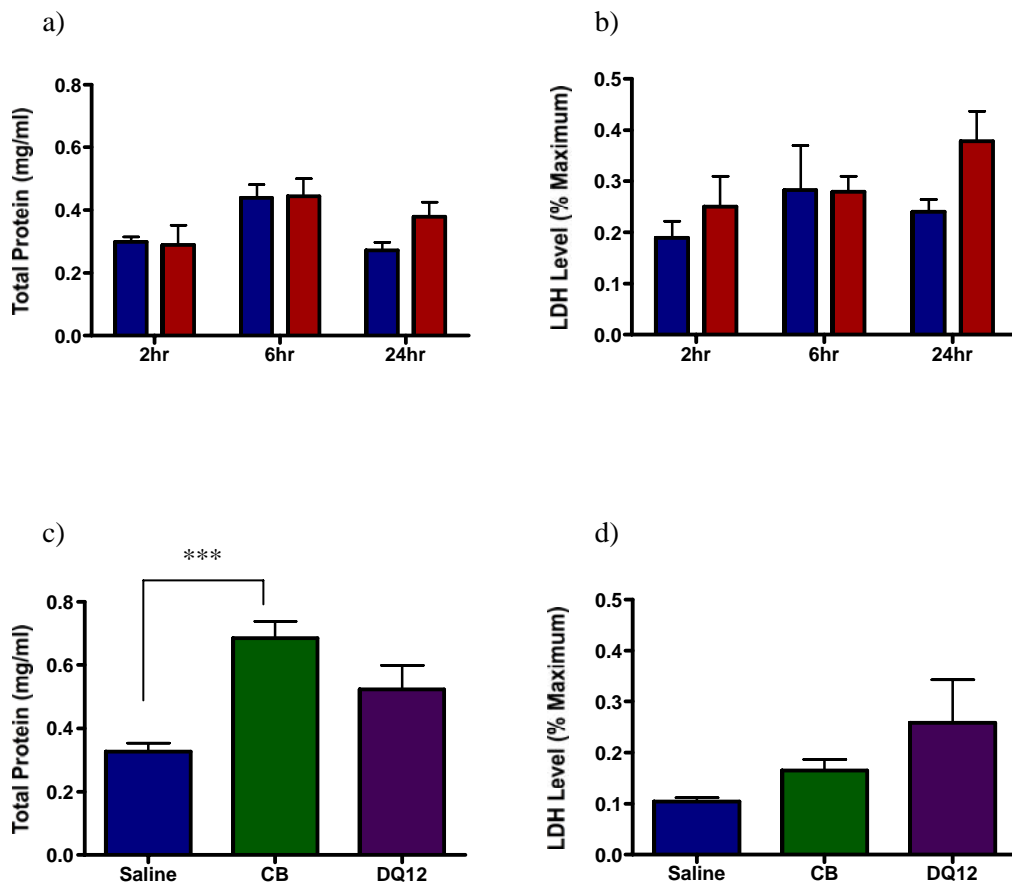
4.3.3.1 Effects of Particulate Instillation on Pulmonary Cytotoxicity and Protein Levels

Measurement of total protein in BAL fluid 2, 6 and 24 hours after instillation showed similar levels in rats treated with saline or with DEP (Figure 4.7a). Increased protein levels were observed 6 hours after instillation when compared to the 2 and 24 hour timepoints, however, this increase was observed in both saline and DEP treated animals, and is therefore likely to be an artefact of the instillation method. Use of control particles (Figure 4.7c) demonstrated that instillation of CB caused a significant increase in total protein in BAL fluid ($P < 0.001$, compared with saline-treated control). In contrast, an apparent increase in total protein in response to DQ12 did not achieve significance ($P > 0.05$).

Analysis of cell death in BAL fluid demonstrated that cytotoxicity was not increased significantly (compared with saline-treated controls) at any of the (2, 6 or 24 hour) time-points after instillation of DEP (Figure 4.7b). An apparent increase in cell death 24 hours after administration did not achieve significance ($P > 0.05$). Similarly, a trend towards increased cell death in BAL samples from rats instilled with CB or DQ12 (Figure 4.7d) did not achieve significance (both $P > 0.05$).

Figure 4.7 - Measurement of Total Protein and Cell Death in Bronchoalveolar Lavage Fluid Following Particle Instillation.

a) Total protein assay of BAL fluid shows no significant differences 2, 6 or 24hrs after instillation of saline (Blue columns) or diesel exhaust particulates (DEP - 0.5mg. Red columns). b) Measurement of cytotoxicity showed no significant differences between saline- and DEP-treated rats at any of the 3 timepoints. c) Total protein assay of BAL fluid 6 hours after instillation of saline (Blue columns), carbon black (CB - 0.5mg; Green columns) or quartz (DQ12 - 0.125mg; Purple columns). Results show a significant increase in total protein in BAL fluid following CB instillation when compared with saline ($P < 0.001$). No significant differences were found between saline and DQ12. d) Measurement of cytotoxicity showed no significant differences between saline, CB- and DQ12-treated groups despite an apparent increase, particularly following DQ12 instillation. Columns represent mean \pm SEM ($n=6$ for all groups). *** $P > 0.001$.



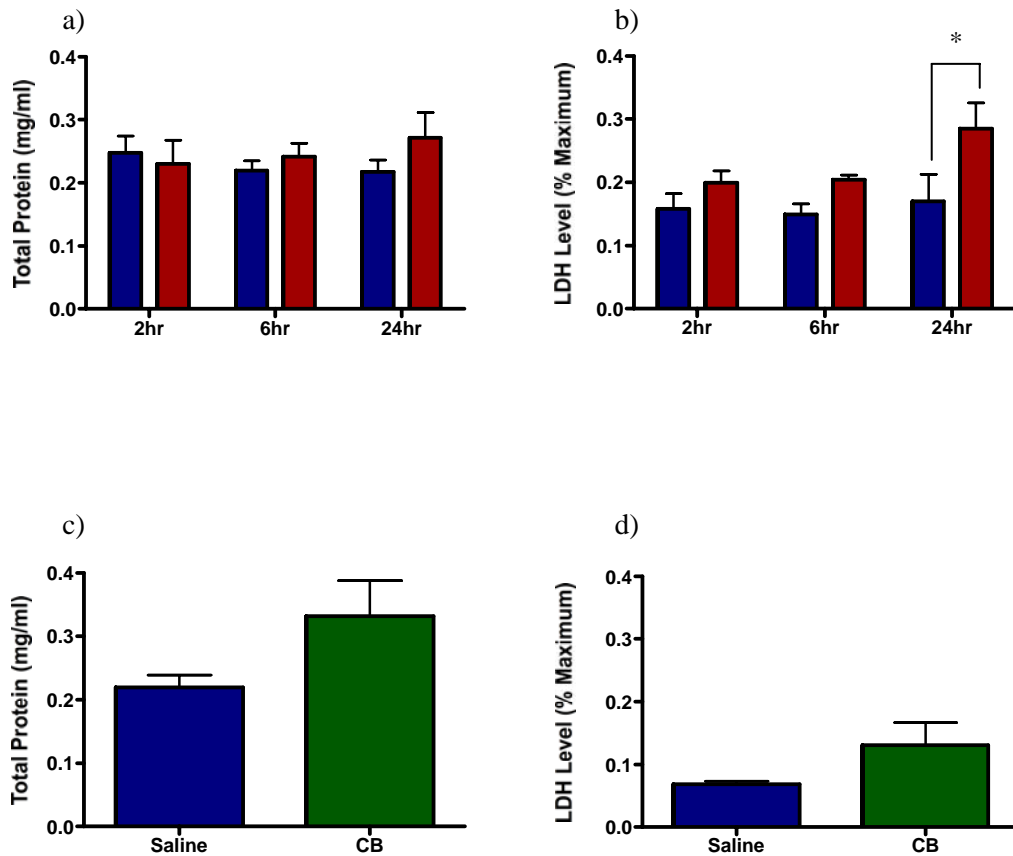
4.3.3.2 Effects of Particulate Injection on Pulmonary Cytotoxicity and Protein Levels

Measurement of total protein in BAL fluid 2 and 24 hours after tail vein injection of saline (Figure 4.8a) demonstrated similar levels to those seen following instillation of saline (Figure 4.7a). Interestingly, the increased protein levels observed 6 hours after instillation of saline were not seen following injection. Injection of DEP had no effect on protein levels in BAL samples at any of the 3 time-points (Figure 4.8a). A trend towards increased total protein was observed in BAL fluid 2 hours after tail vein injection of CB, however, this did not achieve significance ($P=0.086$, Figure 4.8c).

Surprisingly, measurement of cytotoxicity showed a significant increase in pulmonary cell death 24 hours following tail vein injection of DEP ($P<0.05$; Figure 4.8b), but increases at the 2 and 6 hour timepoints did not reach statistical significance. There was no evidence of cell death 2 hours after injection of CB (Figure 4.8d).

Figure 4.8 - Measurement of Total Protein and Cell Death in Bronchoalveolar Lavage Fluid Following Particle Injection.

a) Total protein assay of BAL fluid 2, 6 and 24hrs after tail vein injection of saline (Blue columns) or diesel exhaust particulate (DEP - 0.5mg/kg; Red columns). Injection of DEP did not significantly increase total protein levels. b) Measurement of cytotoxicity showed a significant ($P<0.05$) increase in pulmonary cell death in BAL fluid 24hr after exposure to DEP, but not 2 and 6 hours after exposure. c) Total protein levels in BAL fluid showed a trend ($P<0.1$) towards an increase 2 hours after instillation of carbon black (CB - 0.5mg/kg; Green columns) but this did not achieve significance. d) Cell death was not increased in BAL fluid 2 hrs after tail vein injection of CB. Columns represent mean \pm SEM ($n=6$ for all groups). * $P>0.05$.



4.3.4 Particulate Administration Enhances Platelet Aggregation

4.3.4.1 Effects of Particulate Instillation on Platelet-Monocyte and Platelet-Platelet Aggregation

Using flow cytometry it was shown that platelet-monocyte aggregation was significantly increased 6 hours after DEP instillation ($P < 0.001$ compared with saline-treated controls; Figure 4.9a). In contrast, no increase was seen following instillation of CB or DQ12 (Figure 4.9a). Platelet-platelet aggregation was unaltered 6 hours after instillation of DEP (Figure 4.9b) but was increased following exposure to CB ($P < 0.05$) or DQ12 ($P < 0.01$).

4.3.4.2 Effects of Particulate Injection on Platelet-Monocyte and Platelet-Platelet Aggregation

Flow cytometry experiments demonstrated a significant increase in platelet-monocyte aggregation 2 hours after DEP injection ($P < 0.01$ compared with saline-treated control; Figure 4.10a). A small apparent increase in platelet-monocyte activation after injection of CB did not achieve significance (Figure 4.10a). As with instillation, injection of DEP had no effect on platelet-platelet aggregation at the time-point studied (Figure 4.10b), whereas exposure to the control particle, CB, caused a significant increase ($P < 0.05$ compared to saline).

Figure 4.9 - Effects of Particle Instillation on Platelet-Monocyte and Platelet-Platelet Aggregation.

a) Using flow cytometry it was shown that platelet-monocyte aggregation was increased ($P<0.001$ compared with saline-treated controls; Blue columns) 6 hours after instillation of diesel exhaust particulate (DEP - 0.5mg; Red columns). Instillation of carbon black (CB - 0.5mg; Green columns) or quartz (DQ12 - 0.125mg; Purple columns) had no effect. b) In contrast, platelet-platelet aggregation was unaffected by instillation of DEP, but increased (compared with saline-treated controls) after instillation of CB or DQ12. Results show a significant increase in platelet-platelet aggregates with CB ($P<0.05$) and DQ12 ($P<0.01$) when compared to saline. There was also a significant difference between DEP and DQ12 ($P<0.05$). Columns represent mean \pm SEM ($n=6$ for all groups). * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

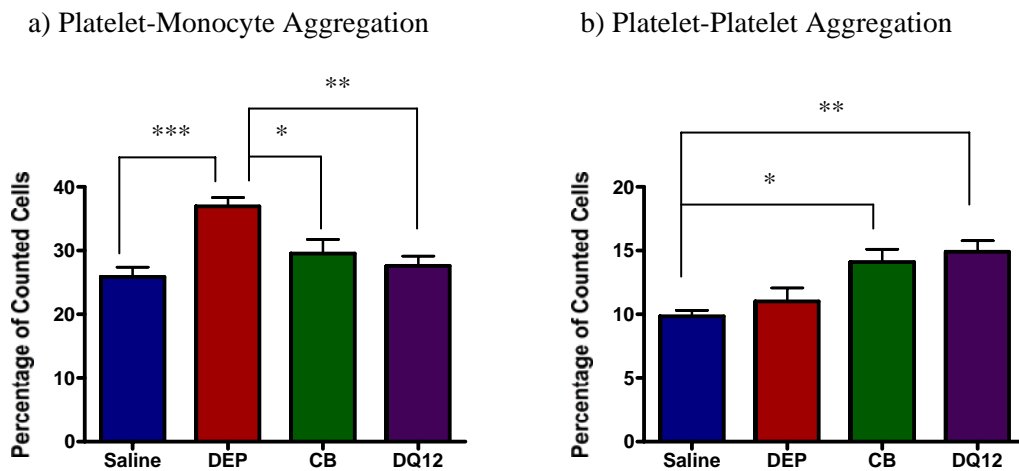
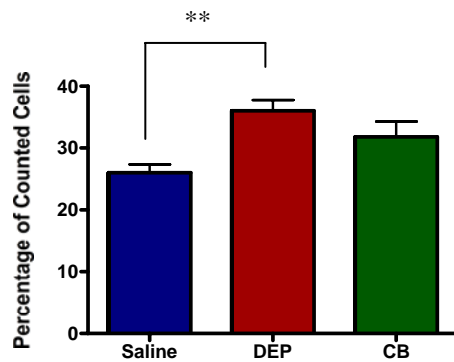


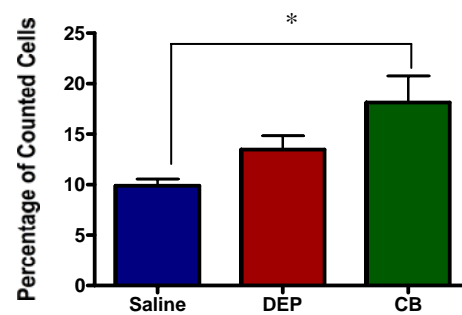
Figure 4.10 - Effects of Particle Injection on Platelet-Monocyte and Platelet-Platelet Aggregation.

a) Using flow cytometry it was shown that platelet-monocyte aggregation was increased ($P < 0.01$ compared with saline-treated controls; Blue columns) 2 hours after tail vein injection of diesel exhaust particulate (DEP - 0.5mg/kg; Red columns). There were no significant differences between carbon black (CB - 0.5mg/kg; Green columns) and saline or DEP. b) In contrast, platelet-platelet aggregation was increased ($P < 0.05$ compared with saline-treated controls). There were no significant differences between DEP and saline or CB. Columns represent mean \pm SEM ($n=6$ for all groups). * $P < 0.05$, ** $P < 0.01$.

a) Platelet-Monocyte Aggregation



b) Platelet-Platelet Aggregation



4.3.5 Effects of Particulate Administration on Pulmonary Cytokine Expression

4.3.5.1 Effects of Particulate Instillation on Markers of Pulmonary Inflammation

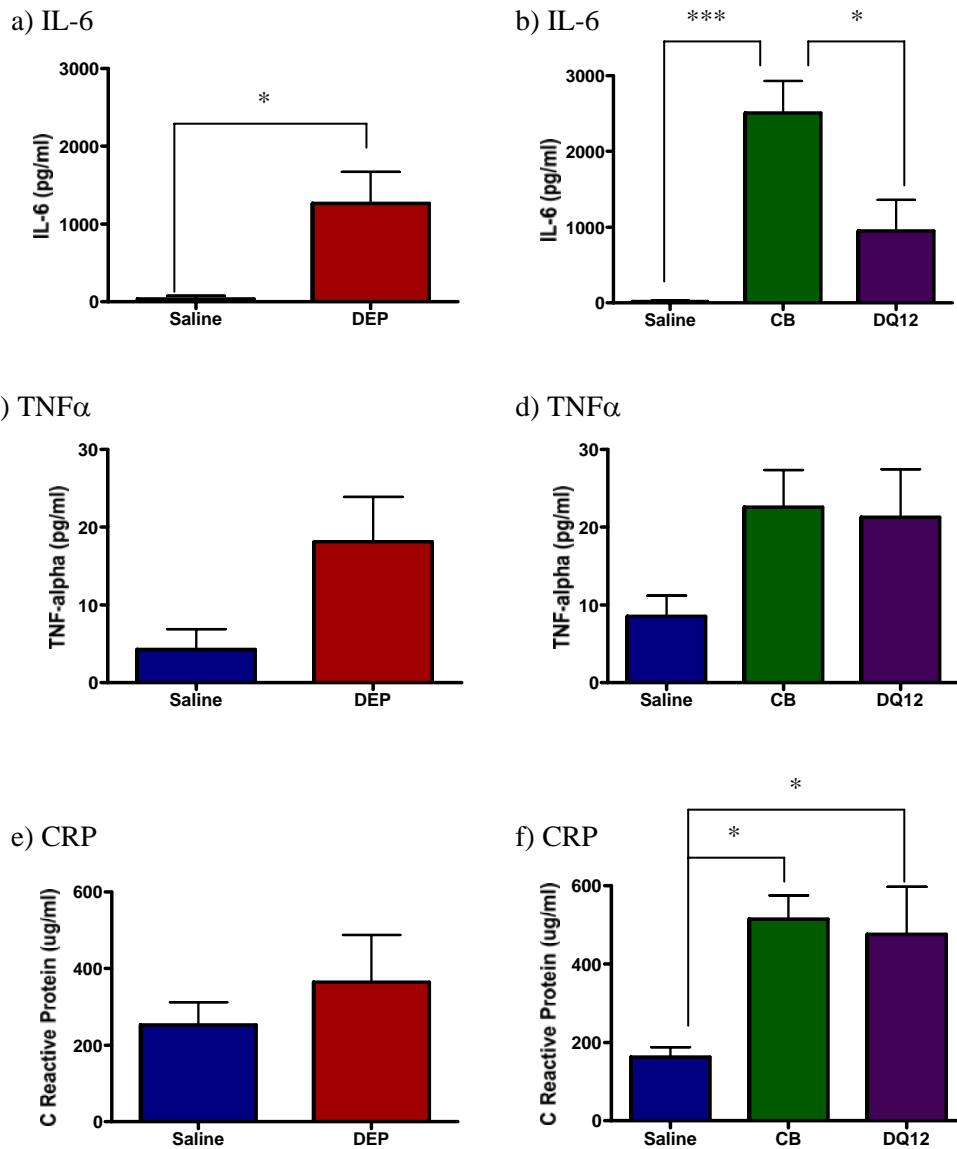
Measurement of IL-6 in BAL fluid samples 6 hours after instillation of test solutions demonstrated that both DEP ($P<0.0001$; Figure 4.11a) and CB ($P<0.001$; Figure 4.11b) produced a significant increase in the levels of this cytokine when compared with saline-treated controls. In contrast, instillation of quartz produced a small increase in IL-6 levels in BAL samples that was not significantly different from controls (Figure 4.11b).

Levels of $\text{TNF}\alpha$ appeared to be elevated (compared to saline-treated controls) 6 hours after instillation of DEP (Figure 4.11c), CB and DQ12 (Figure 4.11d) but these changes were not statistically significant, and were close to the limit of detection for the kit (8pg/ml).

CRP levels were unaltered in BAL fluid samples 6 hours after instillation of DEP (compared with saline-treated controls; Figure 4.11e). In contrast, pulmonary levels of CRP were increased 6 hours following instillation of CB ($P<0.05$) or DQ12 ($P<0.05$ compared with saline-treated controls; Figure 4.11f).

Figure 4.11 - Effects of Particle Instillation on Pulmonary Inflammatory Factors.

a) Results of the interleukin 6 (IL-6) ELISA showed a significant increase in pulmonary IL-6 6 hours after diesel exhaust particulate (DEP - 0.5mg; Red columns) instillation when compared to saline (Blue columns. $P=0.013$). b) Results of the IL-6 ELISA showed a significant increase in pulmonary IL-6 6 hours after carbon black (CB - 0.5mg; Green columns) instillation when compared to saline ($P<0.001$). There was also a significant increase in pulmonary IL-6 following CB when compared to quartz (DQ12 - 0.125mg; $P<0.05$; Purple columns). c) Results of the tumour necrosis factor alpha (TNF α) ELISA showed a non-significant increase in pulmonary TNF α 6 hours after DEP instillation when compared to saline ($P=0.054$). d) Results of the TNF α ELISA showed a non-significant increase in pulmonary TNF α 6 hours after CB and DQ12 instillation when compared to saline ($P>0.05$). e) Results of the C reactive protein (CRP) ELISA showed no significant increase in pulmonary CRP after DEP instillation when compared to saline. f) Results of the CRP ELISA showed a significant increase in pulmonary CRP after CB ($P<0.05$) and DQ12 ($P<0.05$) instillation when compared to saline. Columns represent mean \pm SEM ($n=6$ for all groups). * $P<0.05$, *** $P<0.001$.



4.3.5.2 Effects of Particulate Injection on Markers of Pulmonary Inflammation

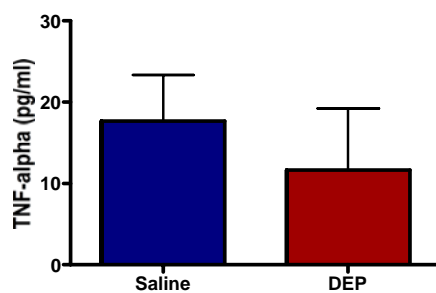
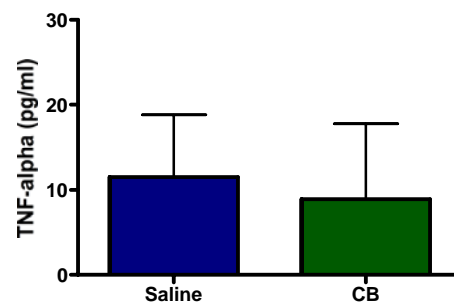
In contrast to samples collected after instillation of saline or test particles (Chapter 4.3.5.1), IL-6 levels were below the limit of detection (125pg/ml) in BAL samples collected 2 hours following injection of test solutions.

Two hours after instillation, levels of TNF α were similar in BAL samples for saline treated controls and in rats exposed to DEP (Figure 4.12a) or CB (Figure 4.12b), however these results were close to the limit of detection for the kit (8pg/ml).

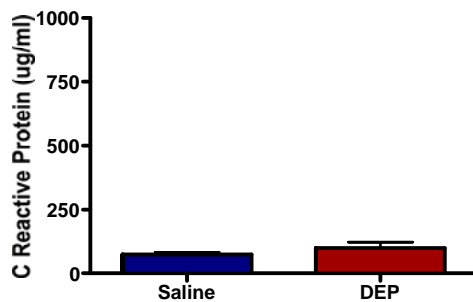
Measurement of CRP in BAL fluid 2 hours after injection of test samples indicated that this marker was unchanged by injection of DEP (Figure 4.12c). A trend ($P < 0.1$) towards increased CRP after injection of CB did not achieve significance. (Figure 4.12d).

Figure 4.12 - Effects of Particle Injection on Pulmonary Inflammatory Factors.

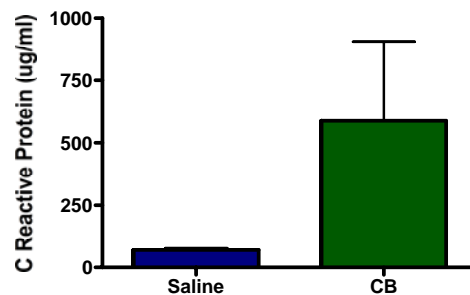
a) Results of the tumour necrosis factor alpha (TNF α) ELISA show no significant increase in pulmonary TNF α after diesel exhaust particulate (DEP - 0.5mg/kg: Red columns) tail vein injection when compared to saline (Blue columns). b) Results of the TNF α ELISA show no significant increase in pulmonary TNF α after carbon black (CB - 0.5mg/kg: Green columns) tail vein injection when compared to saline. c) Results of the CRP ELISA show no significant increase in pulmonary CRP after DEP instillation when compared to saline. d) Results of the CRP ELISA show no significant increase in pulmonary CRP after CB tail vein injection when compared to saline, although there is a trend towards increased pulmonary CRP after CB instillation when compared to saline ($P=0.1336$). Columns represent mean \pm SEM ($n=6$ for all groups).

a) TNF α b) TNF α 

c) CRP



d) CRP



4.3.6 Effects of Particulate Administration on Systemic Inflammation

4.3.6.1 Effects of Particulate Instillation on Markers of Systemic Inflammation

Levels of IL-6 and TNF α in plasma taken 6 hours after instillation of saline, DEP, CB or DQ12 were below the limits of detection (125pg/ml and 8pg/ml for IL-6 and TNF α , respectively) of the assays used.

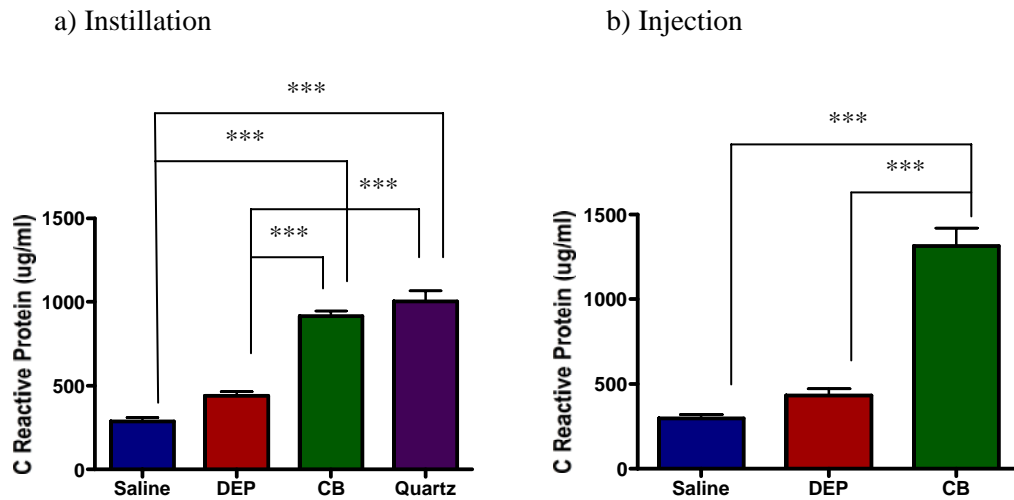
In plasma samples taken 6 hours after instillation of test solutions CRP levels were unaffected by exposure to DEP (Figure 4.13a). In contrast both controls (Figure 4.13a), CB and DQ12, induced an increase in circulating CRP levels ($P < 0.001$ when compared with saline-treated controls).

4.3.6.2 Effects of Particulate Injection on Markers of Systemic Inflammation

As with measurement of IL-6 and TNF α in plasma following instillations, levels of these factors were below the limit of detection (IL-6; 125pg/ml, TNF α ; 8pg/ml) for the relevant ELISAs in plasma samples obtained 2 hours after tail vein injection of saline, DEP or CB (data not shown). In contrast, measurement of plasma concentrations of CRP ELISA showed that CB, but not DEP, produced a significant increase in circulating levels of this protein (Figure 4.13b).

Figure 4.13 - Effects of Particle Instillation and Injection on Systemic Inflammatory Factors.

a) Results of the C reactive protein (CRP) ELISA shows a significant increase in levels of CRP in plasma collected following instillation of carbon black (CB - 0.5mg; Green columns. $P<0.001$) and quartz (DQ12 - 0.125mg; Purple columns. $P<0.001$) when compared to saline (Blue columns). There was no significant difference between diesel exhaust particulate (DEP - 0.5mg; Red columns) and saline, however there were significant increases in plasma CRP following instillation of CB ($P<0.001$) and DQ12 ($P<0.001$) when compared to DEP. b) Results of the CRP ELISA shows a significant increase in levels of CRP in plasma collected following tail vein injection of CB when compared to both DEP ($P<0.001$) and saline ($P<0.001$). There was no significant difference in CRP levels following DEP tail vein injection when compared to saline. Columns represent mean \pm SEM (n=6 for all groups). *** $P<0.001$.



4.3.7 Effects of Particulate Administration on the Fibrinolytic System

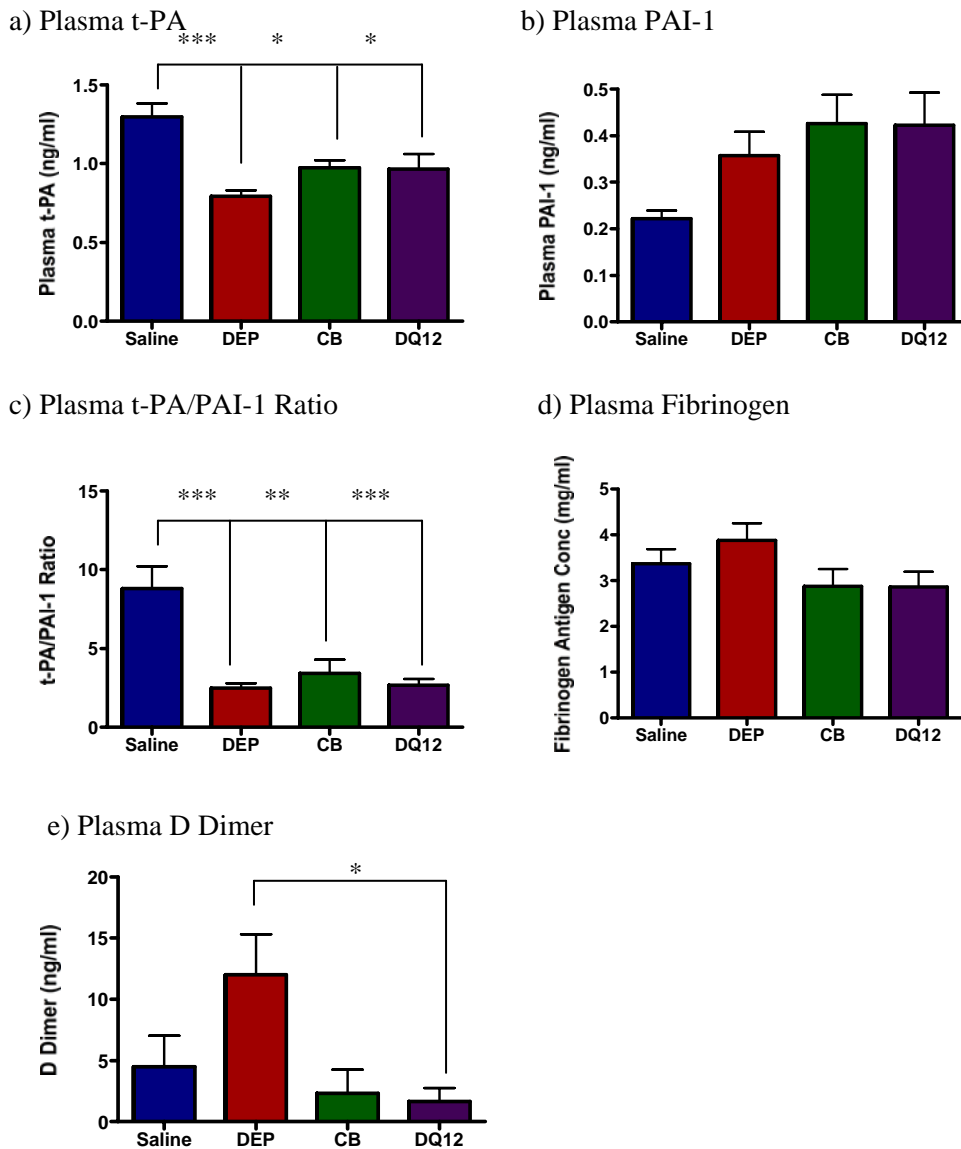
4.3.7.1 Effects of Particulate Instillation on the Fibrinolytic System

Analysis of plasma samples obtained 6 hours after instillation indicated that exposure to DEP caused a significant reduction in levels of t-PA antigen ($P < 0.001$ compared with saline-treated controls; Figure 4.14a). Similar, but smaller, reductions in t-PA antigen were also observed 6 hours after instillation of CB ($P < 0.05$) or DQ12 ($P < 0.05$) (Figure 4.14a). Instillation of each of the three test particulates produced small apparent increases in plasma PAI-1 antigen 6 hours after administration but these changes did not achieve significance (Figure 4.14b). Calculation of the t-PA/PAI-1 ratio (Figure 4.14c) indicated that this was significantly reduced after exposure to DEP ($P < 0.001$), CB ($P < 0.01$) or DQ12 ($P < 0.001$) when compared with saline-treated controls.

Concentrations of plasma fibrinogen (Figure 4.14d) appeared to be elevated 6 hours after instillation of DEP, and reduced after exposure to CB or DQ12. However, none of these changes achieved significance. A significant increase in plasma D dimer (Figure 4.14e) was observed when DEP was compared to quartz, 6 hours after instillation ($P < 0.05$). There were no significant differences between any other groups.

Figure 4.14 - Effects of Particle Instillation on the Thrombolytic System.

a) Results of the tissue plasminogen activator (t-PA) ELISA show a significant reduction in plasma t-PA antigen 6 hours after instillation of diesel exhaust particulate (DEP - 0.5mg; Red columns: $P<0.001$) when compared to saline (Blue columns). There were also smaller, but significant reductions in plasma t-PA antigen following carbon black (CB - 0.5mg; Green columns: $P<0.05$) and quartz (DQ12 - 0.125mg; Purple columns: $P<0.05$) when compared to saline. b) Results of the plasminogen activator inhibitor (PAI-1) ELISA show a general increase in plasma PAI-1 antigen 6 hours after instillation of DEP, CB and DQ12, however, these results were not significant. c) Results of the t-PA/PAI-1 calculation shows significant reductions in the t-PA/PAI-1 ratio 6 hours after DEP ($P<0.001$), CB ($P<0.01$) and DQ12 ($P<0.001$) when compared to saline. d) Results of the fibrinogen ELISA show a general increase in fibrinogen antigen 6 hours after instillation of DEP, with a general decrease observed following instillation of CB and DQ12, however, none of these results are significant. e) Results of the D Dimer ELISA show a general increase in D Dimer antigen 6 hours after instillation of DEP, however there was only a significant difference between DEP and DQ12 instillation ($P<0.05$). Columns represent mean \pm SEM (n=6 for all groups). * $P<0.05$, ** $P<0.001$, *** $P<0.001$.



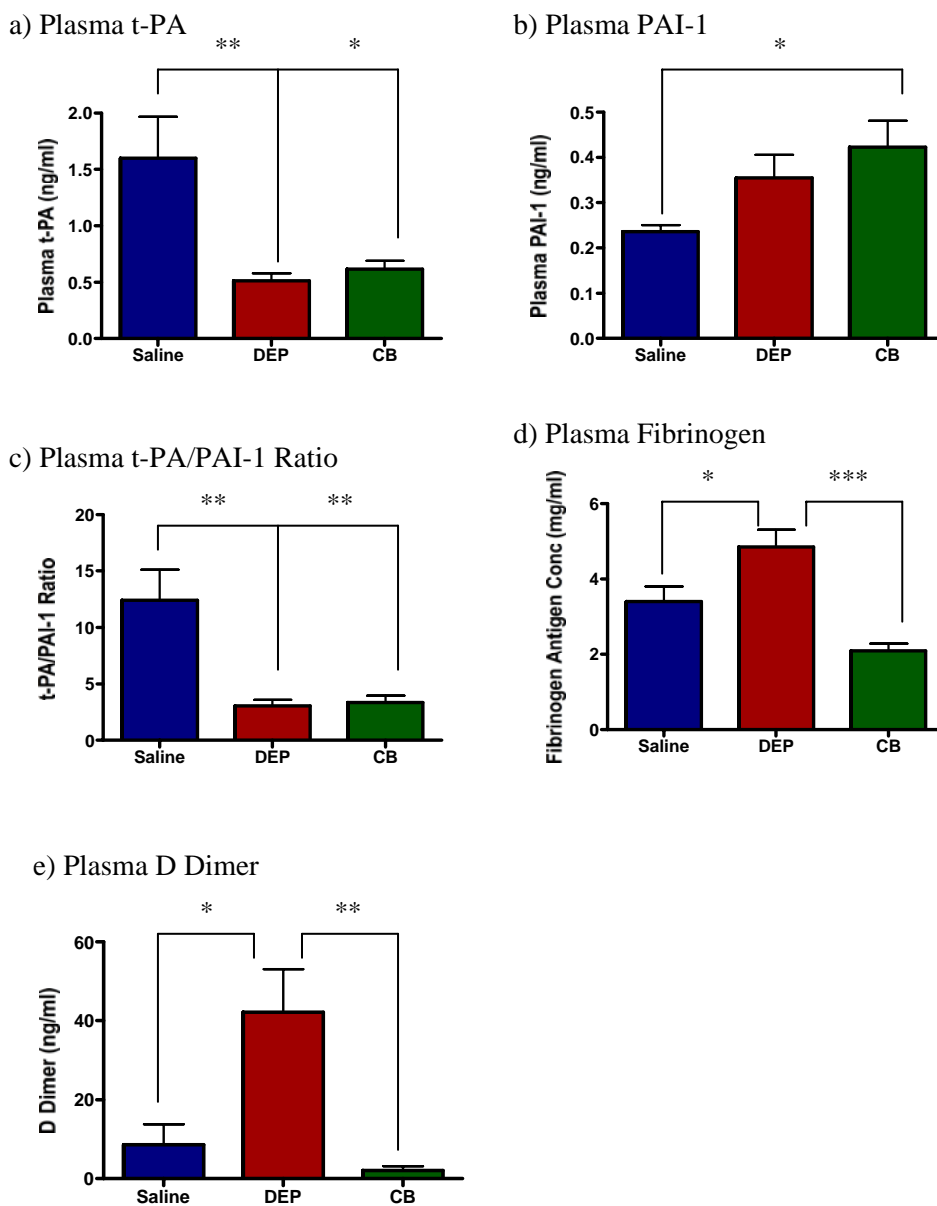
4.3.7.2 Effects of Particulate Injection on the Fibrinolytic System

Analysis of plasma 2 hours following injection of test samples indicated a significant reduction in t-PA antigen (Figure 4.15a) in response to either DEP or CB ($P<0.01$ and $P<0.05$, respectively, when compared with saline-treated control). Plasma concentrations of PAI-1 (Figure 4.15b) antigen were increased 2 hours after tail vein injection of CB ($P<0.05$ when compared with saline-treated controls). A smaller, but non-significant, increase was observed following injection of DEP. Calculation of the t-PA/PAI-1 ratio (Figure 4.15c) indicated that this was significantly reduced in response to both particulate solutions. t-PA and PAI-1 activity ELISAs showed the same trends observed with the antigen ELISAs

Plasma levels of fibrinogen antigen (Figure 4.15d) were increased 2 hours after injection of DEP ($P<0.05$ compared with saline-treated controls) whereas injection of CB produced a small (non-significant) reduction. Significant increases in D dimer antigen (Figure 4.15e) were observed 2 hours after DEP injection when compared with saline ($P<0.05$) and CB ($P<0.01$). No significant change in D dimer was observed between saline and CB.

Figure 4.15 - Effects of Particle Injection on the Thrombolytic System.

a) Results of the tissue plasminogen activator (t-PA) ELISA show a significant reduction in plasma t-PA antigen 2 hours after tail vein injection of diesel exhaust particulate (DEP - 0.5mg/kg: Red columns: $P<0.01$) and carbon black (CB - 0.5mg/kg: Green columns: $P<0.05$) when compared to saline (Blue columns). b) Results of the plasminogen activator inhibitor (PAI-1) ELISA show a non-significant increase in plasma PAI-1 antigen 2 hours after tail vein injection of DEP. There was a significant increase in plasma PAI-1 antigen following CB instillation ($P<0.05$) when compared to saline. c) Results of the t-PA/PAI-1 calculation shows significant reductions in the t-PA/PAI-1 ratio 2 hours after DEP ($P<0.01$) and CB ($P<0.01$) when compared to saline. d) Results of the fibrinogen ELISA show a significant increase in fibrinogen antigen 2 hours after tail vein injection of DEP ($P<0.05$) when compared to saline. There was also a significant reduction in fibrinogen antigen when CB was compared to DEP ($P<0.001$). e) Results of the D Dimer ELISA show a significant increase in D Dimer antigen 2 hours after tail vein injection of DEP when compared to saline ($P<0.05$) and carbon black ($P<0.01$). Columns represent mean \pm SEM ($n=6$ for all groups). * $P<0.05$, ** $P<0.001$, *** $P<0.001$.



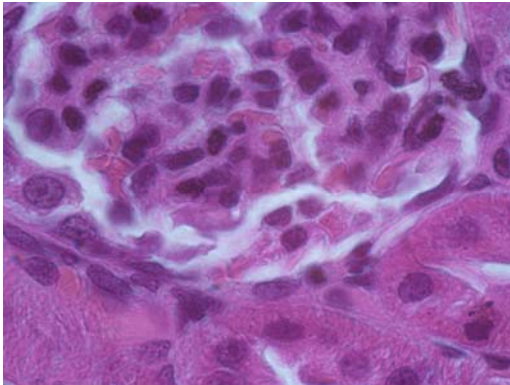
4.3.8 Staining for Evidence of Particulate Translocation and Deposition

There was no evidence of particle accumulation in H&E stained sections of liver (Figure 4.16a) and kidney (Figure 4.16b) taken from rats 6 hours after instillation with DEP. In contrast, similar sections from rats 2 hours after injection with DEP show detectable particulate deposition in the liver (Figure 4.16d) but not in the kidney (Figure 4.16c). Lung sections taken 2 hours after DEP instillation are used as positive controls as large DEP aggregates can be clearly distinguished (Figure 4.16e). DEP can also be easily visualised in the lung 6 hours after instillation (data not shown). No particles were visualised in the liver or kidneys at any other timepoints.

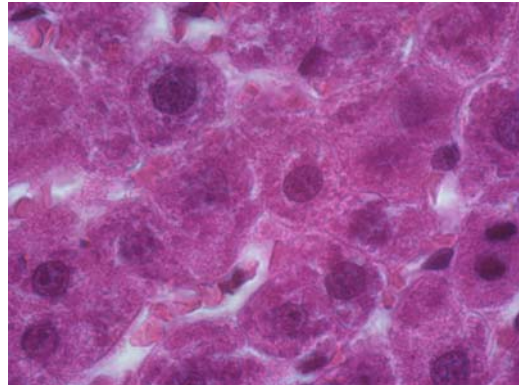
Figure 4.16 - Staining for Evidence of Particle Translocation and Deposition.

a) Kidney section showing glomeruli taken 6 hours after diesel exhaust particulate (DEP) instillation. b) Liver section taken 6 hours after DEP instillation. c) Kidney section showing glomeruli taken 2 hours after DEP injection. d) Liver section taken 2 hours after DEP injection. DEP can be visualised (arrows). e) Positive control. Lung section taken 2 hours after DEP instillation. DEP aggregates can be visualised in some sections (arrows). Pink stain indicates cellular cytoplasm, purple stain indicates cell nuclei. Magnification x1000.

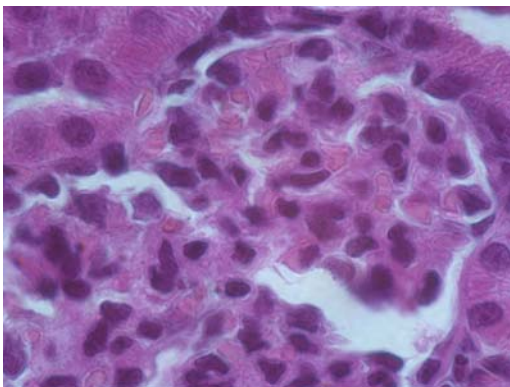
a) Kidney - DEP Instillation, 6hrs



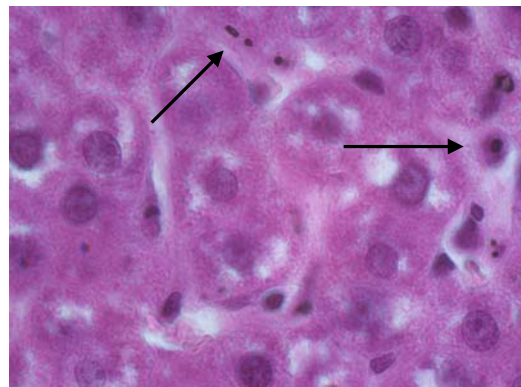
b) Liver - DEP Instillation, 6hrs



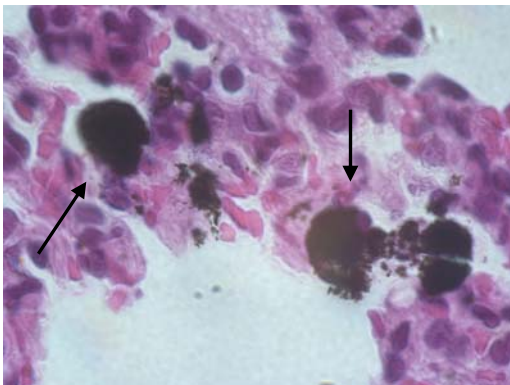
c) Kidney - DEP Injection, 2hrs



d) Liver - DEP Injection, 2hrs



e) Lung - DEP Instillation, 2hrs



4.4 Discussion

The main hypothesis examined in this chapter was that exposure to diesel exhaust particulate (DEP) increases thrombus formation. The experiments described were designed to determine whether:

- (1) this was demonstrable in a rat model of arterial thrombosis,
- (2) the pro-coagulant effect of DEP required administration via the lungs
- (3) pulmonary and systemic inflammation contribute to the effect of DEP, and
- (4) DEP-induced changes in thrombosis were due to alteration in the activity of platelets and the endogenous fibrinolytic system.

The data generated confirmed that exposure to DEP, whether via the lungs or by direct injection into the bloodstream, increased thrombus formation following application of FeCl₃ to the carotid artery. This could not be attributed solely to the development of inflammation as control particles produced inflammatory changes in the lung and the systemic circulation without altering thrombus formation. Accelerated thrombus formation in response to DEP was associated with platelet activation but not with changes in components of the endogenous fibrinolytic system (t-PA, PAI-1) when compared to control particles. Tables 4.1 and 4.2 outline the key results found in this chapter. The methods used in this chapter are well established, and relevant to the area of study; the ferric chloride method has been used successfully to study the effects of various nanoparticles on thrombosis (Kurz *et al.* 1990; Radomski *et al.* 2005; Bihari *et al.* 2009), as has the tail tip (Elg *et al.* 1999; Tanaka *et al.* 1998; Sato *et al.* 1998; Wang & Xu, 2005), flow cytometry to assess platelet function (Radomski *et al.* 2005; Bihari *et al.* 2009) and ELISA to measure pulmonary and systemic inflammation (Ushio *et al.* 1999; Fahy *et al.* 1999; Donaldson *et al.* 2005; Ahn *et al.* 2008). The particulates used in this thesis have also been used in previous animal studies; DQ12 quartz (Duffin *et al.* 2001; Duffin *et al.* 2007), nano-carbon black (Vincent *et al.* 2001; Donaldson *et al.* 2005), and DEP from the National Institute of Standards and Technology (NIST 2975. Nemmar *et al.* 2007a; Nemmar & Inuwa. 2008a; Nemmar *et al.* 2010).

Table 4.1 - Results Summary of Particulate Administration on Thrombus Formation, Fibrinolysis and Platelet Function.

Summary of experiments investigating the effects of particle administration, via intrapulmonary instillation (Ins) and intravenous injection (IV), on thrombus formation via ferric chloride (FeCl₃) administration, platelet aggregation via flow cytometry, and markers of the fibrinolytic system via ELISA, including tissue plasminogen activator (t-PA) antigen, plasminogen activator inhibitor 1 (PAI-1) antigen, t-PA/PAI-1 ratio, fibrinogen antigen and D-dimer antigen. DEP = diesel exhaust particulate, CB = carbon black, DQ12 = quartz particulate, N/C = no change from saline control, NS = change from saline control that was not statistically significant but may produce a physiological result. Timepoint of significant results are indicated after the increase (↑) or decrease (↓) from saline control.

	Thrombus Formation via FeCl₃	Platelet- Monocyte Aggregation	Platelet- Platelet Aggregation	t-PA Antigen	PAI-1 Antigen	t-PA/PAI-1 Antigen Ratio	Fibrinogen Antigen	D-Dimer Antigen
DEP Ins	↑ 6hrs	↑ 6hrs	N/C	↓ 6hrs	↑ 6hrs (NS)	↓ 6hrs	N/C	↑ 6hrs (NS)
CB Ins	N/C	N/C	↑ 6hrs	↓ 6hrs	↑ 6hrs (NS)	↓ 6hrs	N/C	N/C
DQ12 Ins	N/C	N/C	↑ 6hrs	↓ 6hrs	↑ 6hrs (NS)	↓ 6hrs	N/C	N/C
DEP IV	↑ 2hrs	↑ 2hrs	N/C	↓ 2hrs	N/C	↓ 2hrs	↑ 2hrs	↑ 2hrs
CB IV	↑ 2hrs	N/C	↑ 2hrs	↓ 2hrs	↑ 2hrs	↓ 2hrs	N/C	N/C

Table 4.2 - Results Summary of Particulate Administration on Pulmonary and Systemic Inflammation.

Summary of experiments investigating the effects of particle administration, via intrapulmonary instillation (Ins) and intravenous injection (IV), on pulmonary and systemic inflammation, including total pulmonary cell count and neutrophil count, pulmonary protein levels, lactate dehydrogenase (LDH) assay as a measure of pulmonary cytotoxicity, ELISAs for pulmonary levels of the inflammatory markers interleukin-6 (IL-6), tumour necrosis factor alpha (TNF α) and C reactive protein (CRP), and ELISA for systemic CRP levels. DEP = diesel exhaust particulate, CB = carbon black, DQ12 = quartz particulate, N/C = no change from saline control, NS = change from saline control that was not statistically significant but may produce a physiological result. Timepoint of significant results are indicated after the increase (\uparrow) or decrease (\downarrow) from saline control. Results were not achieved for the pulmonary IL-6 antigen ELISA following particle injection, as results were below the limit of detection for the kit.

	Pulmonary Cell Count	Pulmonary Neutrophil Count	Pulmonary Protein Level	LDH Assay	Pulmonary IL-6 Antigen	Pulmonary TNFα Antigen	Pulmonary CRP Antigen	Systemic CRP Antigen
DEP Ins	\uparrow 6hrs	\uparrow 6+24hrs	N/C	N/C	\uparrow 6hrs	\uparrow 6hrs (NS)	N/C	N/C
CB Ins	\uparrow 6hrs	\uparrow 6hrs	\uparrow 6hrs	N/C	\uparrow 6hrs	\uparrow 6hrs (NS)	\uparrow 6hrs	\uparrow 6hrs
DQ12 Ins	\uparrow 6hrs	\uparrow 6hrs	N/C	N/C	N/C	\uparrow 6hrs (NS)	\uparrow 6hrs	\uparrow 6hrs
DEP IV	N/C	N/C	N/C	\uparrow 24hrs	-	N/C	N/C	N/C
CB IV	N/C	N/C	N/C	N/C	-	N/C	\uparrow 2hrs	\uparrow 2hrs (NS)

4.4.1 DEP Increases Thrombus Formation in the Rat Carotid Artery

The primary observation of this chapter was that exposure of healthy rats to DEP reduced the time to vessel occlusion following treatment with FeCl_3 . This pro-thrombotic state was also evident from increases in plasma fibrinogen and d-dimer levels and is consistent with increasing published evidence that exposure to DEP causes an increase in thrombus formation in experimental animals (much of these data coming from Nemmar & colleagues working in Belgium and in the United Arab Emirates). The first demonstration that instillation of DEP (5-500 μg per animal) increased thrombus formation was achieved using hamsters (Nemmar *et al.* 2002a) and has since been confirmed in this species (Nemmar *et al.* 2003; Nemmar *et al.* 2004a), in mice (Cozzi *et al.* 2007; Nemmar *et al.* 2009), and in rats (Nemmar *et al.* 2007). These results have been obtained using NIST diesel particles (as in the present studies), administered in a range of concentrations of DEP either via the lungs (by intra-tracheal instillation) or directly into the systemic circulation (by tail vein injection). There appear to be no instances in the literature of thrombus formation measured in experimental animals after exposure to DEP by inhalation. The concentrations of DEP used for the work reported in this chapter are comparable with those used previously for instillation and injection in experimental animals. For example, instillations were performed using 500 μg DEP/rat compared with 5-500 μg /animal for hamsters (Nemmar *et al.* 2002c; Nemmar *et al.* 2003b; Nemmar *et al.* 2004a) and 15-30 μg /animal for mice (Nemmar *et al.* 2009b). Similarly, intravenous injection was performed using 0.5mg/kg compared with reported doses of 0.01-0.5mg/kg (Nemmar *et al.* 2007; Nemmar & Inuwa. 2008; Nemmar *et al.* 2009a; Nemmar *et al.* 2010) in rats. The timing of the increased thrombotic response was also broadly in keeping with previous work: venous and arterial thrombosis were increased 6 and 24 hours (but not 1 or 3 hours) after instillation of DEP increased in hamsters (Nemmar *et al.* 2002c) whilst injection of DEP altered platelet number 6 hr after administration to rats (Nemmar *et al.* 2010). On balance, therefore, these data suggest that DEP-induced acceleration of thrombus formation is consistent in experimental animals and occurs whether the particulate is administered via the lungs or directly into the circulation. This pro-thrombotic effect is consistent with

clinical studies which indicate that inhalation of DEP alters endogenous fibrinolysis (Mills *et al.* 2005; Mills *et al.* 2007), and increases platelet monocyte activation and *ex vivo* thrombus formation (Lucking *et al.* 2008).

Interestingly, enhanced thrombus formation is not a unique property of DEP. Several investigations have indicated a similar effect with a number of different types of particulate. For instance, increased thrombosis has been reported after instillation of silica particles (hamsters; Nemmar *et al.* 2005), titanium dioxide (TiO₂) nanorods (rats; Nemmar *et al.* 2008b), ambient particulate matter (mice; Cozzi *et al.* 2007), carbon nanotubes (mice; Nemmar *et al.* 2007b) or amine-polystyrene ultrafine particles (hamsters; Nemmar *et al.* 2002a; Nemmar *et al.* 2002b). As demonstrated in this thesis, administration via the lungs is not necessarily required as thrombus formation was enhanced following intravenous injection of quantum dots (mouse; Geys *et al.* 2008) whilst addition of TiO₂ nanorods to rat blood *ex vivo* produced a dose-dependent increase in platelet aggregation (Nemmar *et al.* 2008b). It is clear, therefore, that ultrafine particles other than DEP are capable of increasing thrombus formation. This is consistent with the suggestion in the experiments reported here that ‘control’ carbon black particles could also produce small increases in thrombogenesis 2 hours after injection.

4.4.1.1 DEP Administration Did Not Alter Tail Tip Bleeding Time.

A surprising outcome of these experiments was the failure of the tail tip bleeding time technique to detect increased thrombosis in response to DEP administration. Measurement of tail tip bleeding time, a method of assessing the coagulation cascade and cessation of bleeding, has been previously shown to be platelet dependent (Hodivala-Dilke *et al.* 1999; Nemmar *et al.* 2009a; Nemmar *et al.* 2009b). This technique has been used successfully to demonstrate alterations in clotting following genetic modification of the coagulation cascade (eg. reduced bleeding time in Factor IX knockout mice; Metzner *et al.* 2009) and pharmacological interventions in mice and rats (eg. Momi *et al.* 2001; Kaiser & Markwardt. 1986). More pertinently for this

thesis, it has also been used extensively to assess the effects of DEP (Nemmar *et al.* 2009b) and other nanoparticles (Nemmar *et al.* 2007b; Cozzi *et al.* 2007) on thrombosis in both rats and mice. Furthermore, the pilot data reported previously (Chapter 3) suggested that tail tip bleeding time was reduced following exposure to DEP.

The reasons for this failure of the tail tip transection method can only be speculated upon. It is possible that the extended period of anaesthesia in the preliminary experiments made changes between the groups more apparent, and, consequently, that the use of a shortened period of anaesthesia made the results less significant. There is no literature available that currently supports this theory, however, the length of anaesthesia is the only experimental detail that was changed between the preliminary studies and the experiments described in this chapter. It should be noted, however, that previous studies have shown reduced tail tip bleeding time 24 hours after instillation or injection of DEP (Nemmar *et al.* 2007a; Nemmar *et al.* 2007b). It is more likely that the failure of this technique to detect any differences in clotting was due to its relatively poor sensitivity. Indeed, it has previously been shown that tail tip transection correlates poorly with antithrombotic status in rats (Lavelle & MacIomhair. 1998). Fortunately, this technique was only performed as a back-up method for analysis of coagulation to provide an alternative in case the main model (FeCl₃-induced arterial thrombosis) failed to induce consistent thrombus formation.

4.4.2 Are the Pro-Thrombotic Effects of DEP Caused by Pulmonary Inflammation?

Several previous investigations have suggested that increased thrombosis following exposure to nanoparticles is mediated by the pulmonary inflammation induced by these particles. This has been reported with carbon nanotubes (Nemmar *et al.* 2007b.), silica particles (Nemmar *et al.* 2005), and TiO₂ (Nemmar *et al.* 2008b). Most relevantly, however, a role for pulmonary inflammation has also been suggested following administration of DEP (Nemmar *et al.* 2003b; Nemmar *et al.* 2009b). The investigations described in this chapter controlled for pulmonary

inflammation using CB (a nanoparticle that models the carbon core of DEP and may be able to translocate from the lung to the systemic circulation) and quartz (DQ12), which is known to produce pulmonary inflammation but is considered too large to translocate (Clouter *et al.* 2001; Duffin *et al.* 2001). Potential contamination with bacterial lipopolysaccharide could be excluded since the particulates used have previously been assessed (and found to be below the limit of detection; 0.1 EU/mL) for endotoxin contamination, by others in my group using the limulus amoebocyte lysate (LAL) assay (McQueen *et al.* 2007; Shaw *et al.* 2010).

The results obtained confirmed that DEP instillation did indeed produce pulmonary inflammation that peaked at the same time-point (6 hrs) as accelerated thrombosis. However, it was notable that both control particles (CB and DQ12) produced comparable pulmonary inflammation without altering thrombus formation. This suggests, therefore, that the pro-thrombotic effect of DEP cannot be attributed solely to an inflammatory response in the lungs. This is supported by the evidence obtained from DEP injections, which accelerated thrombus formation without causing a demonstrable pulmonary inflammation. In contrast, and rather unexpectedly, there was some evidence that injection of CB caused some pro-inflammatory changes in the lung (trend towards increased C-reactive protein concentrations in BAL fluid). This has similarities with previous studies reporting that intravenous injection of particles can cause both systemic and pulmonary inflammation (Nemmar *et al.* 2004b; Nemmar *et al.* 2007a; Nemmar & Inuwa. 2008a); although in these cases it was the injected DEP (rather than control particles) that caused pulmonary inflammation.

4.4.3 Are the Pro-Thrombotic Effects of DEP Caused by Systemic Inflammation?

Intravenous injection of nanoparticles has previously been shown to cause systemic inflammation. DEP administration, for example, has been reported to increase circulating levels of IL-6, TNF α and leukotriene B₄ in the rat (Nemmar *et al.* 2009b; Nemmar *et al.* 2010). In the experiments reported here, however, there was very little

evidence of systemic inflammation in response to DEP. Plasma levels of IL-6 and TNF α remained below the limit of detection, even after administration of DEP or CB. Plasma CRP levels were detectable but were unaffected by injection of DEP; interestingly, though, injection of the control (CB) particles did cause a non-significant increase in plasma CRP. This reinforces the conclusion that DEP-mediated pro-thrombotic changes are not simply secondary to systemic inflammation.

In addition to profound pulmonary inflammation, pulmonary instillation of nanoparticles has also been shown to cause inflammatory changes in the systemic circulation (Nemmar *et al.* 2009b; Cozzi *et al.* 2007; Nemmar *et al.* 2004a). This is consistent with the demonstration here that instillation of CB or quartz caused an increase in plasma levels of CRP. Once again, however, instillation of DEP had no observable effect on systemic inflammation. The pro-inflammatory actions induced by CB in the lungs and the systemic circulation were interesting. CB is a 'clean' carbon particle (Heinrich *et al.* 1995) and was initially expected to be less of an inflammatory stimulus than DEP. However, recent work in our lab has shown that CB produces free radicals, at a level comparable to DEP of the same size (Miller *et al.* 2009). Free radical generation could contribute to the inflammatory response following CB instillation. Furthermore, following the start of this work, evidence emerged indicating that CB was capable of producing cytotoxic injury, inflammation and inhibition of growth in endothelial cells (Yamawaki & Iwai. 2006). Parallel studies in my department supported the possibility that CB was more than inert particle, showing that it could induce the release of pro-inflammatory cytokines from macrophages and endothelial cells (although to a lesser extent than DEP; Shaw *et al.* 2010).

4.4.4 Evidence of Particle Translocation

Administration to the blood *ex vivo* has indicated that nanoparticles can directly activate platelets (Nemmar *et al.* 2003a; Nemmar *et al.* 2003b; Lucking *et al.* 2008),

suggesting that pro-thrombotic changes may be the result of particles translocating from the lungs to the systemic circulation. Previous studies have suggested that nanoparticles, including DEP, are capable of translocation; passing from the alveoli into the blood to affect the heart and vasculature directly. Extra-pulmonary translocation of radio-labelled ultrafine polystyrene particles has been shown in rats by measuring the amount of radioactivity collected in the blood and organs following instillation (Chen *et al.* 2006). This study also showed that pre-treatment with lipopolysaccharide induced pulmonary inflammation and markedly increased the rate of translocation: suggesting that inhaled ultrafine particles reach the alveoli and cause inflammation, which makes the alveoli more permeable and allows particles to enter the bloodstream. Similar results have also been shown in healthy human volunteers; radiolabelled ultrafine carbon particles (Technegas) could be detected in the blood of the volunteers one minute after inhalation (Nemmar *et al.* 2002). However, a subsequent study by my group suggested that extra-pulmonary translocation of this particular particle does not occur; the majority of Technegas-labelled carbon particles remained in the lung 6 hours after inhalation, and when signal was detected in the circulation it was attributed to radiolabel that had ‘leached off’ the ultrafine particles before entering the bloodstream (Mills *et al.* 2006). Whether ultrafine diesel particles can pass into the blood still remains unclear. Results presented here did not show any evidence of particle translocation; DEP was only visible in the livers of rats injected with DEP, no particles were visible in the liver or kidneys of rats instilled with DEP. It is possible that particles could accumulate over time at the site of thrombus formation with repeated exposures, producing direct effects on thrombus formation. However, there is no evidence to support that hypothesis in this thesis as particles were not found in the vasculature and, indeed, detection of particle translocation was not a primary aim for the experiments described. It is possible that particles translocating into the vasculature are so small that they might not be detected using standard histological methods. It is also possible that particles in the lungs activate inflammatory cells, which increase thrombosis after translocation into the circulation (Shaw *et al.* 2010). Although this may be partly responsible for the effects observed with DEP, it is unlikely that this is the only explanation given the pro-inflammatory effects of CB and DQ12 (which did

not alter time to occlusion). An alternative possibility is that metals and organic compounds found on the surface of DEP become separated from the particles in the lungs and translocate into the bloodstream, causing the effects on the fibrinolytic system. This could explain the different effects of DEP, CB and quartz.

4.4.5 How does DEP Exposure Increase Thrombus Formation?

The most likely mechanisms to explain the pro-thrombotic response to DEP administration involve changes in platelet function and in the endogenous fibrinolytic system.

4.4.5.1 Exposure to DEP Causes Platelet Activation

Several previous investigations have suggested that the pro-thrombotic response to DEP is the result of platelet activation (Nemmar *et al.* 2002a; Nemmar *et al.* 2003a; Nemmar *et al.* 2003b; Nemmar *et al.* 2004a; Nemmar *et al.* 2005; Nemmar *et al.* 2010). Instillation of non-diesel particulates increased platelet activation (Nemmar *et al.* 2003a) and platelet-granulocyte aggregation in experimental animals (Bihari *et al.* 2009). Platelet activation has been observed *ex vivo* in response to DEP exposure in previous experimental animal studies (Nemmar *et al.* 2002c; Nemmar *et al.* 2004a; Nemmar *et al.* 2008b). Results described in this chapter indicate that platelet activation, assessed by measuring platelet-monocyte aggregation, was increased following either instillation or injection of DEP. This is consistent with results obtained in a previous clinical study into the effects of DEP (Lucking *et al.* 2008), and suggests a pathway through which DEP can increase thrombus formation. It was noted that platelet-platelet aggregation was not increased in animals with enhanced thrombosis, indicating that this measure is a poor predictor of thrombosis in rats *in vivo*, although there is no literature currently available to support or explain this theory.

4.4.5.2 Particle Administration Alters Function of the Endogenous Fibrinolytic System

Clinical investigations performed by researchers in my group have shown that inhalation of DEP impairs the endogenous fibrinolytic system (reducing endothelial t-PA release) in healthy volunteers (Mills *et al.* 2005) and in men with stable coronary heart disease (Mills *et al.* 2007). These effects occurred over a time-course (2-6 hours) consistent with the pro-thrombotic effects seen in the pre-clinical investigations described in this chapter. On the basis of these observations, it was necessary to determine whether altered endogenous fibrinolysis could be implicated in the increased thrombotic response to FeCl₃. Consistent with the clinical studies, DEP administration (whether by instillation or injection) did impair function of endogenous fibrinolysis, with reduced plasma t-PA levels and increased PAI-1. The effects of DEP on the fibrinolytic system have not previously been fully examined using animal models: one report of a DEP-induced inhibition of PAI-1 expression (Furuyama *et al.* 2006) contrasts with the results described here, although ambient particulate matter increased plasma levels of PAI-1 in mice (Cozzi *et al.* 2007). It is apparent, however, that the pro-thrombotic effects of DEP cannot be attributed solely to altered fibrinolysis as both control particles (CB, whether injected or instilled; and DQ12) produced a similar impairment in fibrinolytic function. This suggests that, at best, altered endogenous fibrinolysis is just one component of DEP-mediated acceleration of thrombosis, and acts via a mechanism common to other particulates.

4.4.5.3 Potential Mechanisms of Pro-Thrombotic Effects of DEP

The investigations described in this chapter were not designed to identify definitively which pathways are involved in mediating the pro-thrombotic effects of DEP. As discussed (above), previous investigations have suggested that DEP-mediated thrombotic changes are secondary to inflammation. Indeed it has been shown that the presence of DEP in the lungs leads to the formation of peroxynitrite, which leads to pulmonary inflammation (Ito *et al.* 2000). This would be consistent with the

suggestion that instilled silica particles enhance thrombus formation (Nemmar *et al.* 2004) by stimulating the release of neutrophil elastase into the systemic circulation and, thus, causing platelet activation (Nemmar *et al.* 2005). Support for this mechanism is provided by the demonstration that inhibition of oxidative stress in the mouse lung reduced pulmonary inflammation and reversed the shortening in tail bleeding time caused by DEP instillation (Nemmar *et al.*, 2009, Toxicology). Similarly, inhibition of pulmonary inflammation (using the glucocorticoid dexamethasone or sodium cromoglycate) abolished the pro-thrombotic effects of DEP instillation in hamsters, apparently by preventing degranulation and release of histamine by mast cells (Nemmar *et al.*, 2004, Circulation). The same researchers, however, also demonstrated that an H₁ antagonist which abolished DEP-induced neutrophils influx in the hamster lung did not alter thrombus formation or platelet function.

The studies described in this chapter used control particles to demonstrate that increased thrombosis in response to DEP is not dependent on either pulmonary or systemic inflammation. This is consistent with evidence that particles, including DEP, can directly increase platelet activation and aggregation (Nemmar *et al.* 2003a; Nemmar *et al.* 2003b; Nemmar *et al.* 2010). Thus, the role of inflammation in mediating the pro-thrombotic effects of DEP remains unproven. Indeed, the suggestion that control particles produce milder pro-thrombotic effects and alter the endogenous fibrinolytic system suggest a number of factors contribute to DEP-induced thrombogenicity. Furthermore, it seems likely that some of these effects will be attributable to the nanoparticulate nature of the DEP, and some to specific characteristics (such as surface metals organic chemicals) of diesel combustion-derived particulate.

4.4.6 Conclusions

The main conclusions drawn from the work described in this chapter are that exposure to DEP increases thrombus formation *in vivo*, and that this effect is independent of inflammation. The use of control particles demonstrated clearly that animals with pulmonary and systemic inflammation in excess of that produced by DEP did not exhibit accelerated thrombosis. The issue of particle translocation remains unproven. Injection of DEP confirmed their ability to alter thrombogenesis by direct interaction with the cardiovascular system. Furthermore, the time taken for changes in thrombus formation to occur (2 hrs for injection; 6 hours for instillation) would be consistent with particles translocating from the lungs. There was no direct evidence, however, of such translocation and it seems unlikely that a sufficient number of instilled particles would gain access to the bloodstream to account for this effect. The pro-thrombotic effect of DEP was most clearly attributable to platelet activation. However, there was some evidence control particles could induce a modest increase in thrombus formation, suggesting that the pro-thrombotic action of DEP may be due to a combination of effects general to particles (induction of inflammatory response, alteration of endogenous fibrinolysis) with those specific to DEP (platelet activation). The characteristics of DEP that make them more pro-thrombotic than the control particles (nano-carbon black; quartz) used in this investigation remain to be identified. One of the more likely explanations is that metals and organic compounds associated with the surface of DEP account for their ability to increase thrombosis. This would need to be confirmed in future studies; possibly by administration of the soluble and organic fractions (and the residual carbon particle) obtained by 'washing' DEP (Arimoto *et al.* 2007). Further studies could also include measurement of blood pressure, heart rate and systemic vascular resistance following exposure of animals to DEP. The results described in this chapter provide thorough evidence that DEP can increase thrombus formation via alteration of platelet function independent of systemic or pulmonary inflammation. Further work is now necessary to understand if some of the same processes that have been observed in these animal studies also occur in cultured human endothelial cells.

Chapter 5

The Influence of Diesel Exhaust Particulate on the Thrombolytic System in Cultured Human Umbilical Vein Endothelial Cells

5.1 Introduction

The demonstration that direct injection of diesel exhaust particulate (DEP) induces an acute (2 hours after administration) acceleration in thrombus formation (Chapter 4) is consistent with the hypothesis that particles can interact directly with the cardiovascular system. This indicates that DEP translocating from the lungs may directly alter coagulation pathways. The mechanisms through which DEP achieve this have not, however, been identified, although there is increasing evidence that they can interact directly with the blood to cause platelet activation (Nemmar *et al.* 2003b; Nemmar *et al.* 2010). It is also possible that DEP can interact directly with the arterial wall to alter function of the endogenous fibrinolytic system in vascular endothelial cells. Certainly there is some evidence that organic extracts from DEP reduce PAI-1 expression in rat coronary microcirculation endothelial cells, as a result of increased oxidative stress (Furuyama *et al.* 2006). In addition, the carbon core itself may alter fibrinolytic function as exposure of HUVECs to PM₁₀ reduced expression of t-PA mRNA and protein levels in culture medium (Gilmour *et al.* 2005). This would certainly be consistent with the demonstration (Chapter 4) that intravenous injection of carbon black (CB) produces a change in endogenous fibrinolytic factors similar to that produced by DEP. In order to assess whether DEP can directly alter the fibrinolytic pathway in endothelial cells, the work described in this chapter details the impact of DEP suspensions of markers of fibrinolysis (tissue plasminogen activator (t-PA) and plasminogen activator inhibitor (PAI-1)) in human endothelial cell cultures.

Before cell culture experiments could begin, it was necessary to develop a suitable dispersant which could be used to ensure optimal exposure of endothelial cells to DEP. A dispersant was required which would:

- 1) keep DEP in suspension for prolonged periods of time.
- 2) suspend DEP in required concentrations.
- 3) hold DEP in nanoparticulate form without the formation of aggregates.
- 4) be non-toxic to human endothelial cells in culture.

Previous studies in this field have attempted to solve the dispersant problem by using the detergent Tween (Sagai *et al.* 1993; Ichinose *et al.* 1995; Matsuo *et al.* 2003). Detergents certainly provide a dispersant capable of preventing particles from forming aggregates and from falling out of suspension. However, detergents are also likely to interfere with cultured cells through disruption of cell membranes (Culvenor *et al.* 1982). Surprisingly, none of the groups that used Tween as a dispersant report its effects on cell viability. Indeed, although Matsuo *et al.* (2003) reported that DEP in a Tween suspension induced death of cultured human bronchial epithelial cells, Tween was not suspected of contributing to cytotoxicity. In other studies, detergents have been avoided with dispersion of DEP achieved by sonication in an appropriate cell culture medium (Hashimoto *et al.* 2000; Alfaro-Moreno *et al.* 2007). These investigations, however, rarely document the resultant particulate size or stability of suspensions produced in this manner. Finally, another study assessed the effects of DEP on cell cultures with no explanation of the methods used to generate particulate suspensions (Takizawa *et al.* 2000). Given this apparent lack of research into the dispersants previously used, it was felt that thorough method development was required to allow selection of the optimum dispersant for use with cell cultures.

It has been suggested that organic extracts of DEP can alter fibrinolytic function in endothelial cells (Furuyama *et al.* 2006), possibly by induction of oxidative stress. (Hirano *et al.* 2003). Few studies, however, have assessed the effects of DEP on this process. Furuyama and colleagues (Furuyama *et al.* 2006) showed that organic extracts of DEP reduced cellular excretion of PAI-1 (although with no demonstrable effect on PAI-1 protein within the cells) but did not alter secretion of t-PA (Furuyama *et al.* 2006). This pattern differs from the increase in circulating PAI-1 and decrease in circulating t-PA observed in the *in vivo* response to DEP reported in this thesis (Chapter 4). These differences are possibly explained by the fact that the earlier study (Furuyama *et al.*, 2006) used organic extracts of diesel, rather than DEP. In addition, previous work performed in Edinburgh (Gilmour *et al.* 2005) indicated that CB particles can also reduce t-PA expression and secretion in HUVECs. These results are consistent with the suggestion that different components of the particulate contribute to the effects of DEP.

5.1.1 Hypothesis

The work described in this chapter addressed the hypothesis that exposure to diesel exhaust particulate (DEP) would cause a direct inhibition of the endogenous fibrinolytic system in cultured human umbilical vein endothelial cells (HUVECs).

5.1.2 Aims

The specific aims of the work described in this chapter were:

- i. to develop a dispersant for DEP that was not toxic when applied to HUVECs in culture.
- ii. to determine whether DEP in solution retained the characteristics of nanoparticulates.
- iii. to determine whether exposure to DEP in solution inhibited the endogenous fibrinolytic system in cultured HUVECs.

5.2 Methods

5.2.1 Preparation of DEP Suspensions.

Diesel exhaust particulate (DEP), collected from industrial forklift engine exhausts (SRM 2975), was obtained from the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA). For the purposes of method development, a number of different dispersants were screened in single experiments to assess their suitability for use with cultured cells. Assessment of the suitability of these dispersants was based on their ability to maintain suitable suspensions of DEP in a non-cytotoxic mix. On the basis of this screening, more detailed assessments were made of the sample concentrations and particulate size in suspension in selected dispersants. Each dispersant was tested by using it to make a 1mg/ml suspension of DEP.

5.2.2 Dispersant Development

5.2.2.1 Water

Water was used as a control for testing DEP dispersion. DEP suspensions (1mg/ml) were prepared in deionised water (dH₂O), probe sonicated (Status Homogenisers. Philip Harris Scientific; US70, Lichfield, UK) at 70% power for 5 minutes to ensure dispersion, and centrifuged at 500g for 1 hour in order to remove large DEP aggregates, leaving only nanoparticles in suspension.

5.2.2.2 Tween 20

Tween 20 was also used as a control for testing DEP dispersion. Tween 20 is a detergent, and should, therefore, provide good DEP dispersion without the formation of aggregates. It was not intended for use in cell culture experiments, as it has been previously shown to be cytotoxic (Han *et al.* 2008) and could, therefore, affect the

results of experiments designed to investigate the effects of DEP on cell viability. Tween 20 was made up at concentrations of 0.01 and 1% in dH₂O. This dispersant was then used to make 1mg/ml DEP suspensions, using the method described above (Chapter 5.2.2.1). These suspensions were used only as controls to assess particle size and suspension stability, cultured endothelial cells were not exposed to these suspensions.

5.2.2.3 Cell Culture Medium With or Without Serum and BSA

EGM-2 cell culture growth medium (GM; Lonza Walkersville; Maryland, USA) and EGM-2 medium without added foetal bovine serum (serum-free medium - SFM) both with and without bovine serum albumen (BSA, 1mg/ml - 50µg/ml; Sigma Aldrich; Munich, Germany) were tested as dispersants. BSA is a large protein used to simulate blood composition and which has been used in previous cell culture experiments to stabilise experimental suspensions (Shires *et al.* 1989; Fischer *et al.* 1995). DEP (1mg/ml) suspensions were made in these dispersants as described (Chapter 5.2.2.1).

5.2.2.4 Synthetic Surfactant

Serum-free EGM-2 culture medium with 100µg/ml dipalmitoyl phosphatidylcholine (DPPC. Sigma Aldrich; Munich, Germany), with and without 250µg/ml BSA (Sigma Aldrich; Munich, Germany) was also tested as a dispersant. The DPPC used is a synthetic version of the principal surfactant in the human lung (Fisher & Dodia. 1997). This solution was heated to 60°C for 10 min and sonicated using a probe sonicator for 30 min (85% power, 5 cycles) in order to fully disperse DPPC. This dispersant was used to make DEP suspensions (1mg/ml), as described (Chapter 5.2.2.1). Collaboration with another lab in the department subsequently suggested

that DPPC has cytotoxic effects on some cell types (C. Poland, Centre for Inflammation Research. Unpublished data).

5.2.2.5 Phosphate Buffered Saline With or Without BSA

Phosphate buffered saline (PBS), and PBS + 0.5mg/ml BSA (Sigma Aldrich; Munich, Germany), were also tested as dispersants. These experiment were conducted after preliminary assessment of dispersants, which found that a 0.5mg/ml BSA solution was most effective for dispersal of 1mg/ml DEP. DEP suspensions (1mg/ml) were prepared using this dispersant as described (Chapter 5.2.2.1).

5.2.3 Analysis of DEP Suspensions

Following preparation of DEP suspensions, the top 80% of the supernatant was aspirated off and used in measurements of absorbance (as a measure of DEP concentration), and mean particle diameter.

5.2.3.1 Absorbance Spectra Measuring DEP Concentration in Suspension

DEP suspensions (1mg/ml) were diluted to a 1/50 concentration with dH₂O and analysed by spectrophotometer (SmartSpec 3000. Bio-Rad; Hertfordshire, UK) measuring absorbance at 500nm, to give a comparative estimation of the amount of diesel in suspension.

5.2.3.2 Analysis of Particle Size in Suspension

Following spectrophotometric analysis, the 1/50 dilutions of the diesel suspensions were assessed using a particle size analyser (90 Plus particle size analyser.

Brookhaven Instruments Corporation; NY, USA), which uses laser scatter to calculate mean particle diameter.

5.2.3.3 Confirmation of DEP Concentration in Suspensions

The concentration of DEP in individual suspensions was also assessed by spinning aliquots (1ml) in a heated vacuum centrifuge overnight, along with 1ml samples of SFM + 0.5mg/ml BSA. The dry weights of the SFM + BSA samples were subtracted from the dry weights of the DEP suspensions to give the mass of DEP/ml. This was then used to calculate DEP concentrations in all future experiments.

5.2.4 Endothelial Cell Culture

Human umbilical vein endothelial cells (HUVECs - Promocell; Heidelberg, Germany) were cultured at 37°C in 75cm³ flasks using endothelial cell culture growth medium (EGM-2 BulletKit - basal medium containing human epidermal growth factor, hydrocortisone, gentamicin, amphotericin B, foetal bovine serum, vascular endothelial growth factor, human fibroblast growth factor B, insulin-like growth factor 1, ascorbic acid and heparin - Lonza Walkersville; Maryland, USA). When cells had grown to confluence they were passaged by first removing the culture medium and then washing the cells twice with 3ml HEPES-buffered saline solution (Reagent Pack Subculture Kit - Lonza Walkersville; Maryland, USA). Cells were then incubated with 3ml trypsin/EDTA solution (Reagent Pack Subculture Kit - Lonza Walkersville; Maryland, USA) at 37°C for 5 minutes, and the sides of the flask were gently tapped to lift cells from the culture surface. Trypsin neutralising solution (5ml; Reagent Pack Subculture Kit - Lonza Walkersville; Maryland, USA) was then added to the cells to prevent lysis. The cell suspension was split equally between four new flasks, 15ml culture medium was added and the flasks were incubated at 37°C. Cells up to passage 6 (P6) were used for further experiments. For

endothelial cell exposures EGM-2 medium without added foetal bovine serum (serum-free medium - SFM) + 0.5mg/ml BSA was used to disperse DEP.

5.2.5 Assessment of Cell Death.

5.2.5.1 Trypan Blue Exclusion

HUVECS (200,000 cells/well in 2ml GM) were plated onto 6 well plates and incubated (2 hours, 37°C) to allow adhesion to the well surface. DEP suspensions were then added to the wells to give final DEP concentrations of 20 and 60µg/ml. Control wells contained dispersant in GM without DEP. Cells were incubated in the presence of DEP suspensions for 2, 6, 16 and 24 hours under standard culture conditions. After the appropriate exposure time, HUVECs were photographed and assessed for viability using Trypan Blue exclusion (Cook and Michell, 1989). Briefly, the HUVECs were washed and trypsinised and an aliquot (10µl) of the cell suspension was mixed with 90µl trypan blue. Live cells actively exclude the stain and appear white when examined microscopically. Dead cells cannot exclude the stain and, therefore, appear dark blue. Cells were counted using a haemocytometer; the number of dead cells was expressed as a percentage of total cells counted.

5.2.5.2 Lactate Dehydrogenase Assay

HUVECs (50,000 cells/well in 100µl GM) were seeded onto 96 well plates and incubated (2 hours, 37°C) to allow adhesion to the well surface. The cell culture medium was then removed and replaced with 100µl DEP suspension in the appropriate dispersant diluted in SFM to give final concentrations of 10, 20, 40, 60 and 80µg/ml. Control wells received 100µl SFM alone. Cells were left to incubate for 2, 6, 16 and 24 hours under standard culture conditions. Positive controls were generated by adding hydrogen peroxide (final concentrations 1µM - 1mM) or Tween 20 (final concentrations 0.1% - 50%) to induce cell death. LDH release was

determined in a colorimetric assay by measuring absorbance at 490nm (cytotoxicity detection, LDH assay kit; Roche Diagnostics). Cytotoxicity was calculated as a percentage of maximum and spontaneous LDH release, as determined by high and low control wells. Background absorbance was taken into account by measuring the absorbance at 490nm of SFM and each diesel concentration in the absence of endothelial cells.

5.2.6 Assessment of DEP on the Release of Fibrinolytic Factors from HUVECs

5.2.6.1 Assessment of DEP on Basal Release of Fibrinolytic Factors

The impact of exposure to DEP on the activity of components of the endogenous fibrinolytic system was assessed using the dispersant/DEP combination selected from pilot investigations on cell viability. Briefly, HUVECs were cultured in GM and then exposed to DEP dispersed in SFM containing 0.5mg/ml BSA (made up to a final volume of 15ml with SFM; final DEP concentrations were 10, 20, 50, 100 and 150mg/ml) for 2, 6, 16 and 24 hours, in order to assess the effects of DEP on constitutive release of fibrinolytic factors.

5.2.6.2 Assessment of DEP on Stimulated Release of Fibrinolytic Factors

Double exposure experiments were designed to test the effects of DEP on stimulated release of fibrinolytic factors. Cells were first incubated with either 100µg/ml DEP (prepared in SFM containing 0.5mg/ml BSA, added to 15ml SFM), or serum free medium alone, for 16 hours. Cells were then washed and exposed to a range of thrombin concentrations (0.1, 0.5 and 1U/ml in SFM with SFM used as a second control) for 24 hours.

5.2.6.3 Collection and Storage of Endothelial Cell Samples Following DEP Exposure

At the end of both experiments, aliquots of medium (1ml) were taken and stored at -70°C for later analysis of tissue plasminogen activator (t-PA) and plasminogen activator inhibitor (PAI-1) levels. RNA from these cells was also extracted and stored at -70°C for analysis of these fibrinolytic factors using polymerase chain reaction (PCR).

5.2.7 Reverse Transcriptase Polymerase Chain Reaction Analysis of HUVECs

Reverse transcriptase polymerase chain reaction (RT-PCR) was used (see Chapter 2.7.4.1) to assess the phenotype of HUVECs in culture.

5.2.7.1 RNA Extraction

Cultured cells were trypsinised, centrifuged (1000rpm, 5min) and the supernatant removed and discarded. RNA extraction was achieved using the TRIzol extraction method (Chapter 2.7.3.1). Following the extraction process, samples were vortexed and centrifuged (7,500g, 5 min, 4°C), the supernatant discarded and the RNA pellets allowed to air dry (room temperature, 5 min). Pellets were then re-suspended in autoclaved 0.1% diethylpyrocarbonate (DEPC)-treated water (50µl) by incubating at 60°C for 10 min.

5.2.7.2 RNA Integrity

RNA concentration was determined using a GeneQuant spectrophotometer reading at 320nm. The integrity of the extracted RNA was verified by denaturing agarose gel electrophoresis (Chapter 2.7.3.1). RNA was then visualised on a UV trans-illuminator at 254nm and photographed using a Mitsubishi P93 video printer.

5.2.7.2 Reverse Transcription

Reverse transcription of extracted RNA was performed using a Promega A3500 RT system. RNA (1µg/ml) was added to the Promega Mastermix (containing 10x Buffer, MgCl₂, Oligonucleotides, dNTPs and RNase inhibitor at a ratio of 2:4:1:2:1). Reverse transcriptase enzyme was added and samples were incubated at 42°C for 30 min, 99°C for 5 min and 4°C for 10 min in an Eppendorf 5331 thermal cycler. At this time two negative controls were added, one containing no reverse transcriptase enzyme, and a second containing no RNA.

5.2.7.4 Polymerase Chain Reaction for Tie2

RT-PCR was conducted to show RNA expression in HUVECs up to P6. Primers for Tie2, an endothelial cell specific tyrosine kinase receptor for angiopoietin (Davis *et al.* 1996), were designed using Gene Jockey software to show that HUVECs retained characteristics of endothelial cells up to P6 (Partanen *et al.* 1992). Primer sequences are shown in Table 5.1. Following reverse transcription, PCR was performed using a Promega M5661 Taq Bead Hot Start Polymerase kit. cDNA samples were added to the Promega Mastermix (containing 10x Buffer, MgCl₂, dNTPs, forward primer, reverse primer and nuclease free H₂O at a ratio of 5:3:1:2:2:32). Samples were run in an Eppendorf 5331 thermal cycler at 95°C for 45 secs, then 35 cycles of 90°C for 45 secs, 60°C for 30 secs, 72°C for 90 secs, then finally 72°C for 5 min and 4°C for 10

min. Samples were run on a 1.2% agarose gel along with a 100 bp DNA ladder (Promega Corporation; Southampton) and visualised using a UV trans-illuminator at 254nm.

5.2.7.5 Polymerase Chain Reaction for t-PA and PAI-1

RNA was extracted from cultured HUVECs exposed to DEP in the experiments described previously, and RT-PCR was performed for t-PA, PAI-1 and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as described (above). Primer sequences are given in Table 5.1.

Table 5.1 - Reverse Transcription Polymerase Chain Reaction Primers.

RT-PCR primer sequences and band sizes for endothelial tyrosine kinase receptor for angiopoietin (Tie2), tissue plasminogen activator (t-PA), plasminogen activator inhibitor (PAI-1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

	Forward Primer	Reverse Primer	Band Size
Tie2	TCA CTC CAG TAT CAG CTC AAG GG	CAG CTG GTT CTT CCC TCA CGT T	300bp
t-PA	ATC TTT GCC AAG CAC AGG AGG TCG	CTT CAG CCG CTC CGA ATA GAA AGG	410bp
PAI-1	AGC ACG GTC AAG CAA GTG GAC T	GGC AGT TCC AGG ATG TCG TAG TAA	325bp
GAPDH	CCA CCC ATG GCA AAT TCC ATG GCA	TCT AGA CGG CAG GTC AGG TCC ACC	600bp

Table 5.2 - Real Time Polymerase Chain Reaction Primers.

Real-time PCR primer sequences for tissue plasminogen activator (t-PA), plasminogen activator inhibitor (PAI-1), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β -Actin.

	Forward Primer	Reverse Primer
t-PA	CGG GTG GAA TAT TGC TGC T	CTT GGC TGG CTG CAA CTT
PAI-1	CTC CTG GTT CTG CCC AAG T	CAG GTT CTC TAG GGG CTT CC
GAPDH	GCT AGG GAC GGC CTG AAG	GCC CAA TAC GCC AAA TCC
β Actin	CCA ACC GCG AGA AGA TGA	TCC ATC ACG ATG CCA GTG

5.2.8 Quantitative Real-Time Polymerase Chain Reaction

RNA was extracted from HUVECs exposed to DEP as described (Chapter 2.7.4.1), reverse transcribed to cDNA and stored at -70°C until real-time PCR was performed. Real time primers (shown in Table 5.2, Invitrogen) were diluted to 100µM using TE (Tris EDTA buffer solution; Fluka BioChemika, Switzerland). Fluorescent probes were added to each set of forward and reverse primers to produce a Mastermix (0.1µl each) along with 5µl Roche probe master, 2.75µl RNase free distilled water (Both from LightCycler 480 probes master kit; Roche, Burgess Hill, UK) and 2µl of cDNA (previously diluted 1 in 8 using RNase free water). Samples were analysed using a Roche LightCycler 480 and corresponding software, and analysed by comparing the unknown samples to serial 1:2 dilutions for each set of primers.

5.2.9 Analysis of the Impact of DEP on t-PA and PAI-1 Release by HUVECs

Supernatants sampled from cell culture experiments (see above) were assessed for t-PA antigen and activity using a t-PA Combi Actibind ELISA kit (Technoclone Ltd. Surrey, UK), and PAI-1 activity using a Technozym PAI-1 Actibind ELISA kit (Technoclone Ltd. Surrey, UK). Full experimental design and kit details can be found in Chapter 2.7.2.

5.2.10 Statistics

Data are expressed as mean \pm SEM. Results were analysed using GraphPad Prism software. Comparison of 2 groups was performed using an unpaired two-tailed t-test. Comparison of 2 groups for 2 experimental conditions was achieved using a two-way ANOVA with a Bonferroni post-test. Significance was assumed when $P < 0.05$.

5.3 Results

5.3.1 Effects of Dispersants on Particle Suspension and Mean Particle Diameter

5.3.1.1 Initial Dispersant Development

A range of dispersants was prepared (as described in Chapter 5.2.1) and used to suspend DEP at 1mg/ml. The original dispersants included dH₂O, Tween 20 (0.1 and 1% in dH₂O), serum-free EGM-2 culture medium (SFM) containing a range of BSA concentrations (50µg/ml - 1mg/ml), SFM containing 100µg/ml DPPC and SFM containing 100µg/ml DPPC and 250µg/ml BSA. The absorbance spectra of 1:10 dilutions of the DEP suspensions showed that Tween 20 provided the highest retention of DEP in suspension. Good suspension was also achieved using BSA in medium as the dispersant, especially at 0.5mg/ml (Figure 5.1a). Measurement of particle diameter showed that culture medium containing BSA maintained low mean particle diameter; again, particularly at 0.5mg/ml (Figure 5.1b).

5.3.1.2 Assessment of Growth Medium and Bovine Serum Albumen as Dispersants

Absorbance spectra of 1 in 10 dilutions of 1mg/ml DEP suspended in a range of dispersants including phosphate buffered saline (PBS), SFM and full EGM-2 growth medium (GM) both with and without BSA (0.5mg/ml). The results of this assay showed that BSA improved particle suspension when added to PBS or SFM. GM provided good suspension both with and without BSA (Figure 5.2a). Mean particle diameter analysis was carried out on the suspensions that retained DEP to a measurable level in the previous experiment (Figure 5.2b). Results show that SFM + BSA, GM and GM + BSA all retained particles with a low mean diameter (<50nm). This indicates that all of these suspensions are suitable for use in future cell culture experiments.

Figure 5.1 - Dispersion and Mean Particle Diameter of DEP in a Range of Dispersants.

The suitability of different dispersants for DEP was assessed by measuring absorbance, to estimate diesel exhaust particulates (DEP) concentration, and by measuring particle size to determine whether particle aggregation was occurring in suspensions. DEP suspensions (1mg/ml) were prepared in a range of dispersants; all BSA and DPPC dispersants were originally made up in serum-free EGM-2, Tween 20 was made up to the correct concentrations in dH₂O and used along with dH₂O as controls. All suspensions were then diluted 1:10 using serum free EGM-2 for analysis, following centrifugation at 500g for 1 hour. a) Most dispersants, with the exception of medium containing the lowest concentrations (50-100µg/ml) of BSA, allowed production of stable, concentrated suspensions of DEP. b) Most dispersants (once again, excepting the lowest concentrations of BSA) produced suspensions of DEP with mean particle diameters below 200nm. Results were not statistically analysed due to low group number (n=3). AU - arbitrary units.

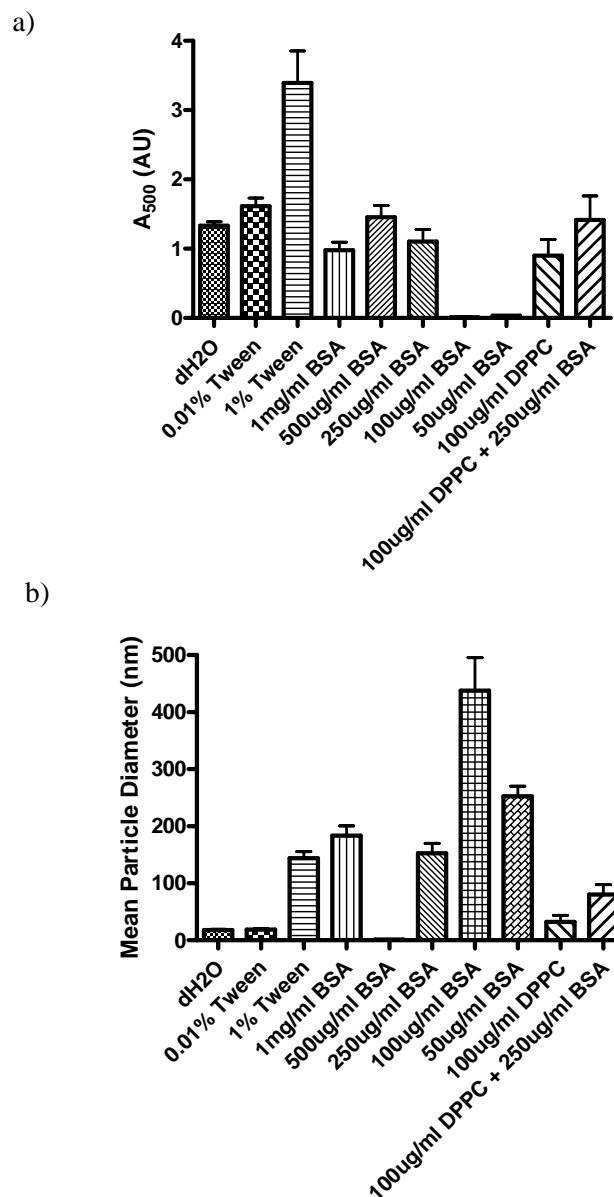
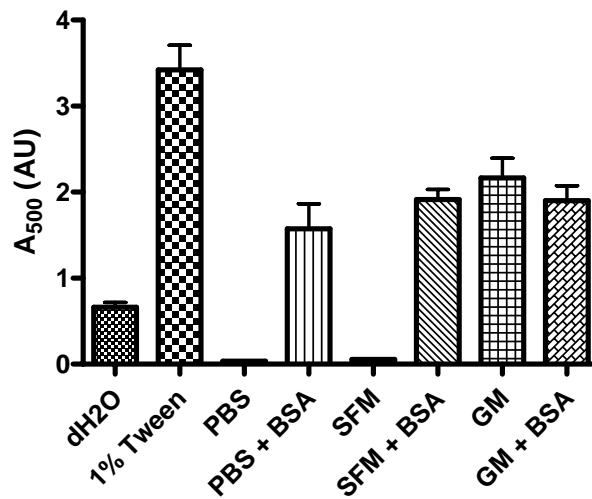


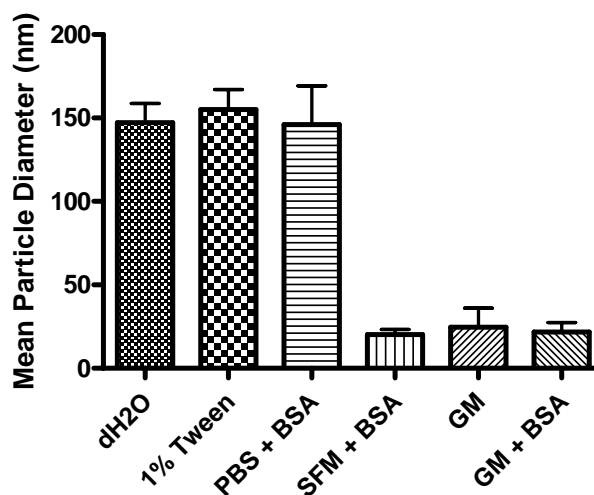
Figure 5.2 - Comparison of the Influence of Growth Factors and Bovine Serum Albumen on Suspension of DEP in EGM-2.

The effect of growth factors and bovine serum albumen (BSA) on formation of suspensions of DEP in EGM-2 was assessed by measuring absorbance (to estimate the concentration of DEP) and mean particulate size (to assess particle aggregation). DEP suspensions (1mg/ml) in the relevant dispersant were diluted 1:10 using serum free EGM-2, following centrifugation at 500g for 1 hour. a) Measurement of absorbance indicated that phosphate buffered saline (PBS) and serum free EGM-2 (SFM) failed to hold DEP in suspension. Addition of bovine serum albumen (BSA) or foetal bovine serum to EGM-2 (GM) maintained DEP in suspension to a similar degree. Distilled water and 1% Tween 20 were used as controls. b) Mean particle diameter remained below 200nm for all dispersants but was dramatically reduced (to ~25nm) by the addition of BSA to PBS or of growth factors (GM) to EGM-2 (SFM). Statistical analysis was not performed due to small group size (n=3).

a)



b)



5.3.2 Investigation into the Cytotoxic Effects of Dispersants

5.3.2.1 Cytotoxic Effects of Tween 20

Preliminary investigations were carried out to examine the cytotoxic effects of a 2 hour exposure to Tween 20 (0.1, 0.5, 1 and 1.5% in serum free medium) on HUVECs using trypan blue exclusion. Over the range examined, the number of live endothelial cells decreased with increasing Tween 20 concentrations (data not shown). However no dead endothelial cells were observed at any of these concentrations (data not shown). The absence of dead cells could be due to the detergent effects of Tween 20; upon microscopic examination of the HUVECs tested the cells were found to be rounder and less adherent to the cell culture plate.

5.3.2.2 Cytotoxic Effects of Synthetic Surfactants

Preliminary investigations were carried out to examine the cytotoxic effects of a 24 hour exposure to DPPC (100µg/ml) and DPPC (100µg/ml) + BSA (250µg/ml). Results suggested that there was some reduction in the number of live cells and an increase in the number of dead cells following exposure to these dispersants (data not shown). These changes were subtle, and no statistical analysis was possible due to the low group number (n=1). However, results from other in the group (Centre for Inflammation Research, University of Edinburgh) also suggested that DPPC was toxic to cells in culture. Consequently, use of DPPC as a dispersant was not pursued further.

5.3.3 Confirmation of the Ability of the Selected Dispersant to Maintain DEP in Suspension Without Particle Aggregation

Suspensions of DEP (1mg/ml) were prepared using EGM-2 GM or SFM containing BSA (0.5mg/ml). Suspensions were then diluted using SFM to a range of concentrations likely to be used in cell culture experiments. Absorbance spectra of

these suspensions confirmed a linear relationship between sample absorbance and concentration (Figure 5.3a). Absorbance spectra were similar whether suspensions were made using SFM or GM. Measurement of mean particle diameter showed that, over the concentration range examined, both SFM and GM kept DEP particle size below 200nm (Figure 5.3b). These results indicate that either dispersant is suitable for preparing suspensions of DEP for use with cultured cells. SFM containing BSA was chosen as the dispersant for subsequent experiments as it provided good particle dispersion in the absence of serum and, therefore, could be used without stimulating endothelial cell proliferation. The amount of DEP in suspensions made using the SFM/BSA combination was confirmed by oven drying 1mg/ml samples. This showed that ~0.652mg/ml DEP was left in a stable suspension following centrifugation (n=6, data not shown). This value was used in all subsequent experiments to calculate DEP concentrations before application to HUVECs.

5.3.4 Effects of DEP on Endothelial Cell Viability

5.3.4.1 Trypan Blue Exclusion Assay for Cell Death

Trypan blue exclusion showed a trend towards reduced cell viability dependent on incubation time and DEP concentration (Figure 5.4). Statistical analysis was not possible on these results due to the low group number (n=3). This experiment was discontinued due to evidence that DEP adhering to the cell surface was producing false positives.

5.3.4.2 Lactate Dehydrogenase Assay for Cell Death

Measurement of LDH demonstrated that incubation with DEP (Figure 5.5c) had little or no effect on HUVEC viability, when compared with positive controls such as Tween 20 (0.1, 1, 5 or 10%; Figure 5.5b) and hydrogen peroxide (1, 10, 100, 500 or 1000µM; Figure 5.5a).

Figure 5.3 - Characteristics of DEP Suspensions Prepared Using EGM-2 Containing Growth Factors of Bovine Serum Albumin.

Suspensions of DEP (10-100 μ g/ml) prepared in serum free EGM-2 medium containing bovine serum albumen (BSA) or foetal calf serum (GM) were compared for particulate concentration and size. Suspensions were prepared (1mg/ml) in EGM-2 GM (blue), or serum-free EGM-2 medium containing 0.5mg/ml BSA (red), and diluted in serum-free EGM-2 to give a range of experimental concentrations. a) Absorbance spectra demonstrate a linear relationship between absorbance and concentration of DEP suspensions. Results were identical in suspensions generated using SFM/BSA and GM. b) Mean particle diameters remained below 200nm for all concentrations and were similar whether suspensions were prepared in GM (blue) or in SFM/BSA (red). Data points represent mean \pm SEM. Statistical analysis was not performed because of low group numbers (n=4).

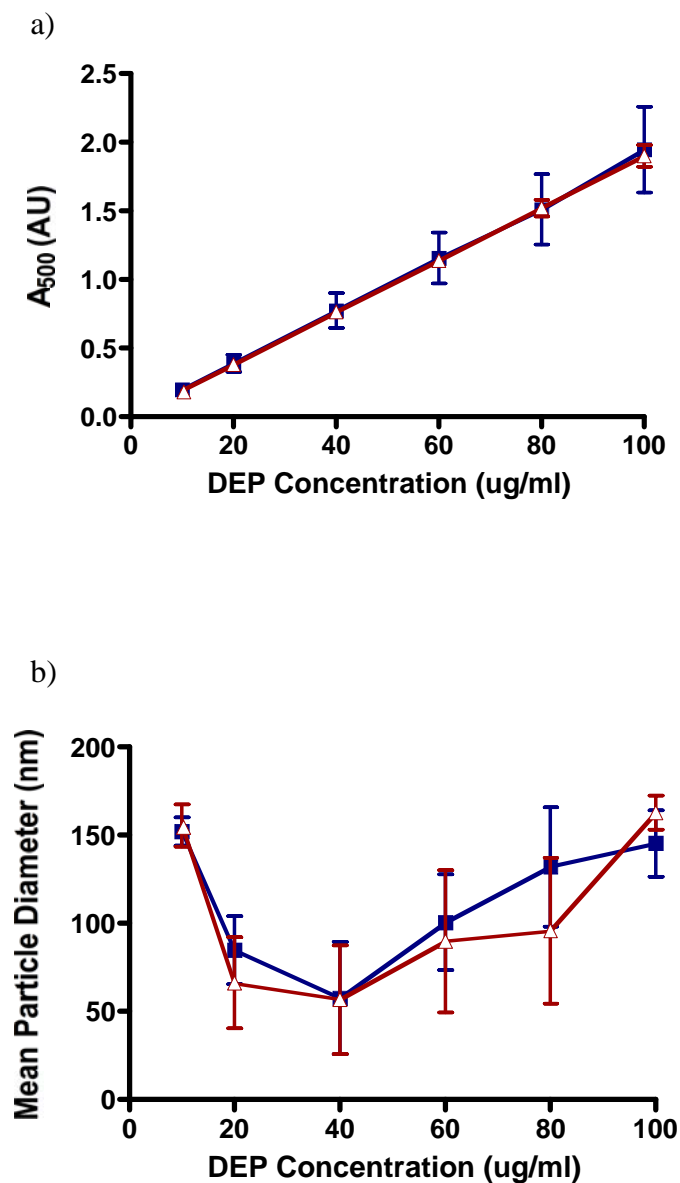


Figure 5.4 - Influence of DEP on Endothelial Cell Viability - Trypan Blue Exclusion Assays.

Trypan blue exclusion assays for cell death following exposure of HUVECs to a) 20 and b) 60 μ g/ml DEP for 2, 6, 16 and 24 hours. Results suggest a time- and concentration-dependent decrease in cell viability. Columns represent mean \pm SEM. Data were not statistically analysed because of small group sizes (n=3).

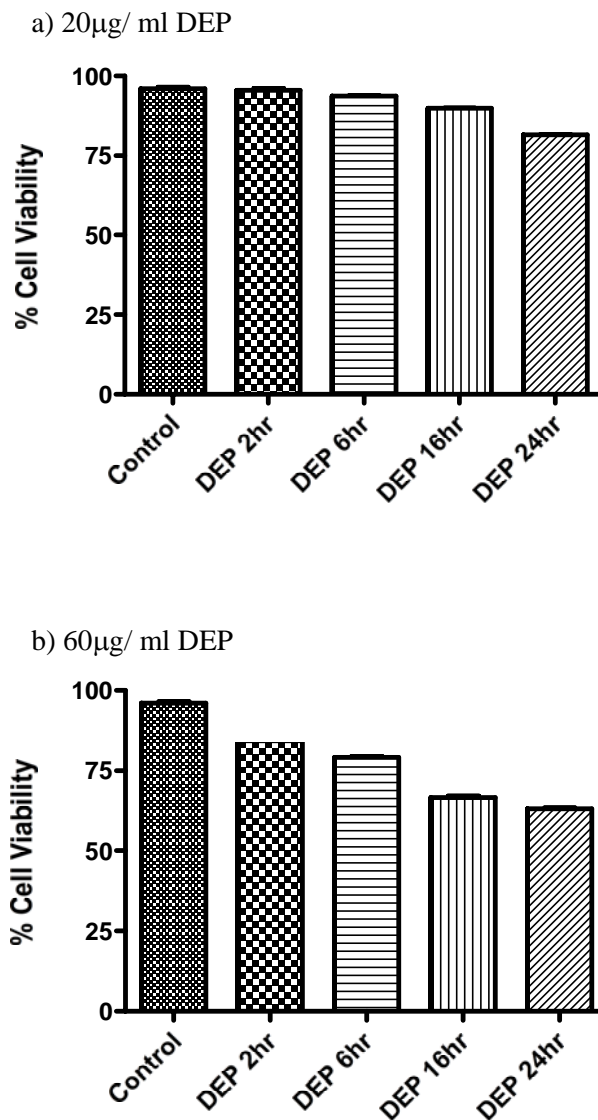
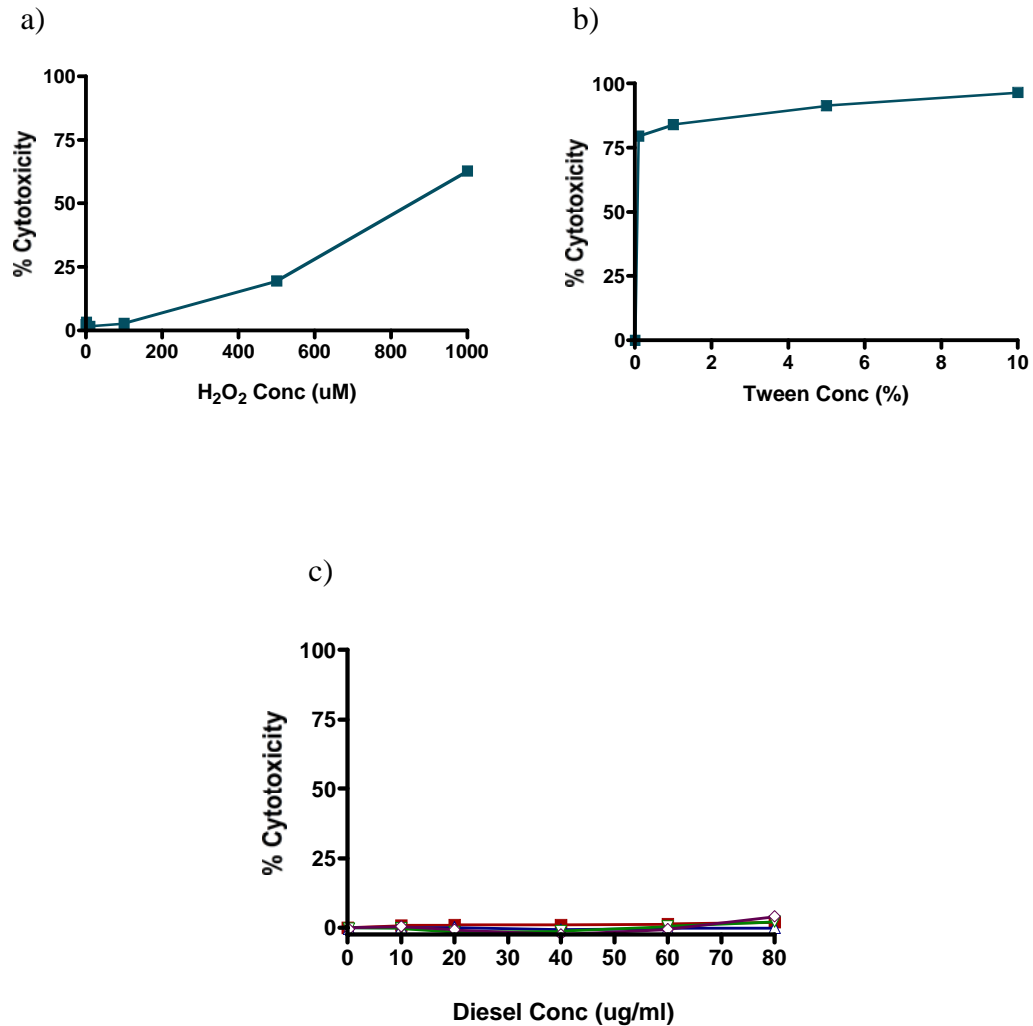


Figure 5.5 - DEP Suspensions do not Reduce EC Viability - LDH Assay.

a) HUVEC exposure to H_2O_2 (1, 10, 100, 500 or 1000 μM , 24 hours) produced extensive, concentration-dependent cell death (n=1). b) HUVEC exposure to Tween 20 (0.1, 1, 5 or 10%, 24 hours) produced almost total cell death at all concentrations (n=1). c) HUVEC exposure to DEP (10, 20, 40, 60 or 80 $\mu g/ml$) for 2 (red), 6 (blue), 16 (green) and 24 (purple) hours produced no increases in cell death (n=6).



5.3.5 The Influence of DEP Suspensions on the Endogenous Fibrinolytic System in Cultured Endothelial Cells

5.3.5.1 Expression of Tie-2 in HUVECs: Reverse Transcription Polymerase Chain Reaction

RNA was extracted from 2 samples of HUVECs and RT PCR performed using primers for the endothelial cell marker Tie2 (Primer sequence shown in Table 5.1). Bands 300bp in size were detected in HUVECs at P6, suggesting conserved expression of Tie2 (Figure 5.6). No bands were visible in the two negative controls; the first of which did not contain the reverse transcriptase enzyme necessary for RT PCR (-RT), and the second of which contained RNase free water instead of sample RNA (H₂O). This experiment suggests that HUVECs up to P6 retain characteristics of endothelial cells and can be used for subsequent experiments.

5.3.5.2 Reverse Transcription Polymerase Chain Reaction for t-PA and PAI-1.

RT PCR was performed in preliminary experiments exposing HUVECs to DEP concentrations up to 80µg/ml over 2 to 24 hours. RT PCR for PAI-1 showed bands (325bp) in all samples, but there appeared to be no significant change in band density over the exposure range. An apparent increase in band brightness towards the centre of the gel was probably a photographic artefact (Figure 5.7). RT PCR was also conducted for t-PA following the same exposures. However, no bands were detected for any of the exposures, even though 2 different primer sets were tested (results not shown). This could indicate that the primers did not function correctly, or that RT-PCR is not a sensitive enough technique to pick up basal levels of t-PA. It was, therefore, decided that real-time PCR would be used to study the direct effects of DEP on HUVECs.

Figure 5.6 - RT PCR showing Tie2 expression in P6 HUVECs.

RT PCR gel showing 300bp bands in two separate samples (samples 1 and 2) of passage 6 (P6) HUVECs, indicating the presence of the endothelial cell marker Tie2. Bands were not present in either of the negative control wells; -RT contained no reverse transcriptase enzyme necessary for PCR, H₂O contained RNase free water instead of sample RNA. HUVEC, Human umbilical vein endothelial cells; RT-PCR, reverse transcriptase polymerase chain reaction.

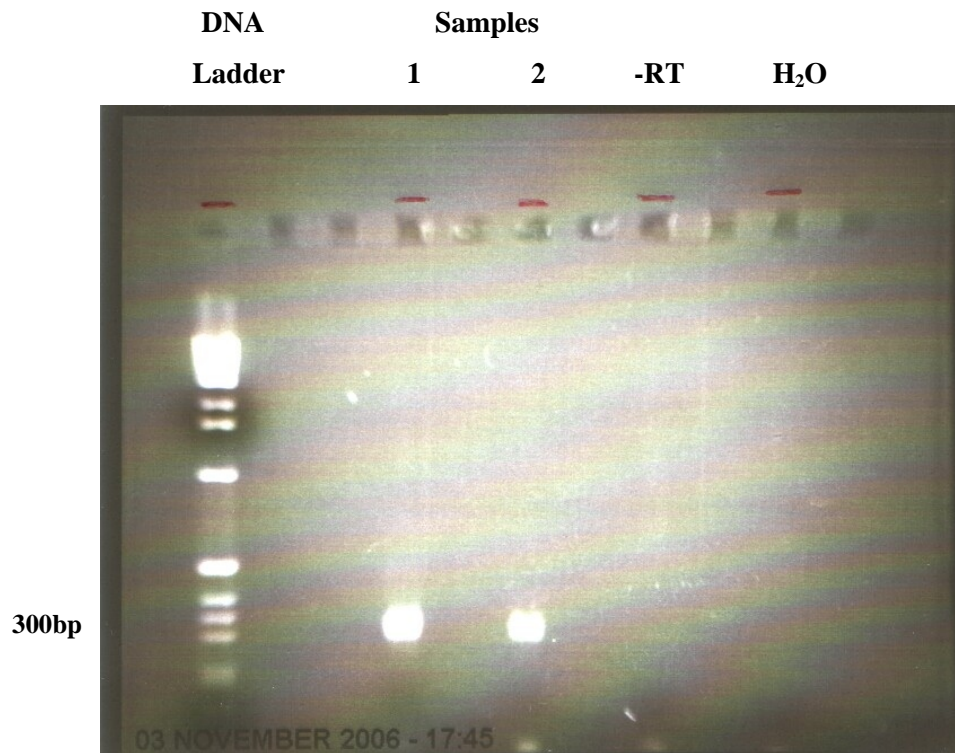
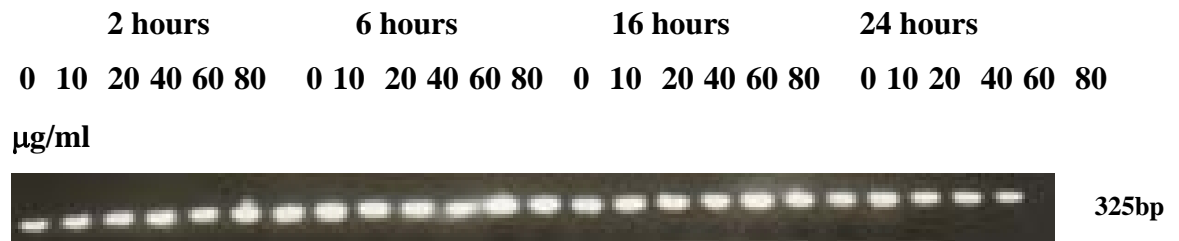


Figure 5.7 - Effects of HUVEC Exposure to DEP on PAI-1 Expression:

Analysed by RT PCR.

Reverse transcriptase polymerase chain reaction (RT-PCR) gel showing bands for plasminogen activator inhibitor (PAI-1) at 325bp in human umbilical vein endothelial cells (HUVECs). Diesel exhaust particulate (DEP) concentration and incubation time are shown above each band. Results suggest no change in PAI-1 expression following DEP exposure.



5.3.6 Effects of DEP Exposure on Basal Expression of Fibrinolytic Factors in Human Endothelial Cells.

Quantitative real-time PCR analysis of HUVECs following exposure to DEP indicates a concentration-dependent, time-dependent decrease in t-PA expression (Figure 5.8c). In contrast, there appeared to be a concentration-dependent, time-dependent increase in PAI-1 expression following exposure to DEP (Figure 5.8d). After 2 and 6 hour DEP incubations PAI-1 expression in cells exposed to 150µg/ml DEP was approximately 3 times higher than basal levels. However, this effect was less apparent when incubations were extended to 16 and 24 hours. Data were quantified with reference to internal controls, GAPDH and β -Actin, which did not vary in response to incubation time or exposure to DEP. Statistical analysis of these data was not performed due to small (n=3) group numbers.

Key observations were investigated by increasing group sizes for two of the DEP exposures to allow statistical analysis; 6 and 24 hour exposures to 150µg/ml DEP were compared with control exposures for the same time periods. In both cases it was confirmed that exposure to DEP reduced t-PA expression (Figure 5.9a) but increased expression of PAI-1 (Figure 5.9b).

Figure 5.8 - Effects of DEP Exposure on Basal Expression of Fibrinolytic Factors in Human Endothelial Cells.

Expression of tissue plasminogen activator (t-PA) and plasminogen activator inhibitor in endothelial cells (HUVECs) after exposure to diesel exhaust particulate (DEP; 10-150 μ g/ml) for up to 24 hours. Expression of positive controls, GAPDH (a) and β -actin (b) were unaffected following exposure to DEP. In contrast, exposure to DEP produced a time-dependent, concentration-dependent decrease in t-PA expression (c) but a time-dependent, concentration-dependent increase in PAI-1 expression (d). Interestingly, this increase in PAI-1 expression was blunted after longer (16, 24 hour) exposures. Symbols represent mean \pm SEM (n=3) after incubation for 2 (red), 6 (blue), 16 (green) or 24 (purple) hours. Expression of t-PA and PAI-1 was calculated by comparison with positive controls (GAPDH and β -actin) expression. Statistical comparisons were not made due to small group size.

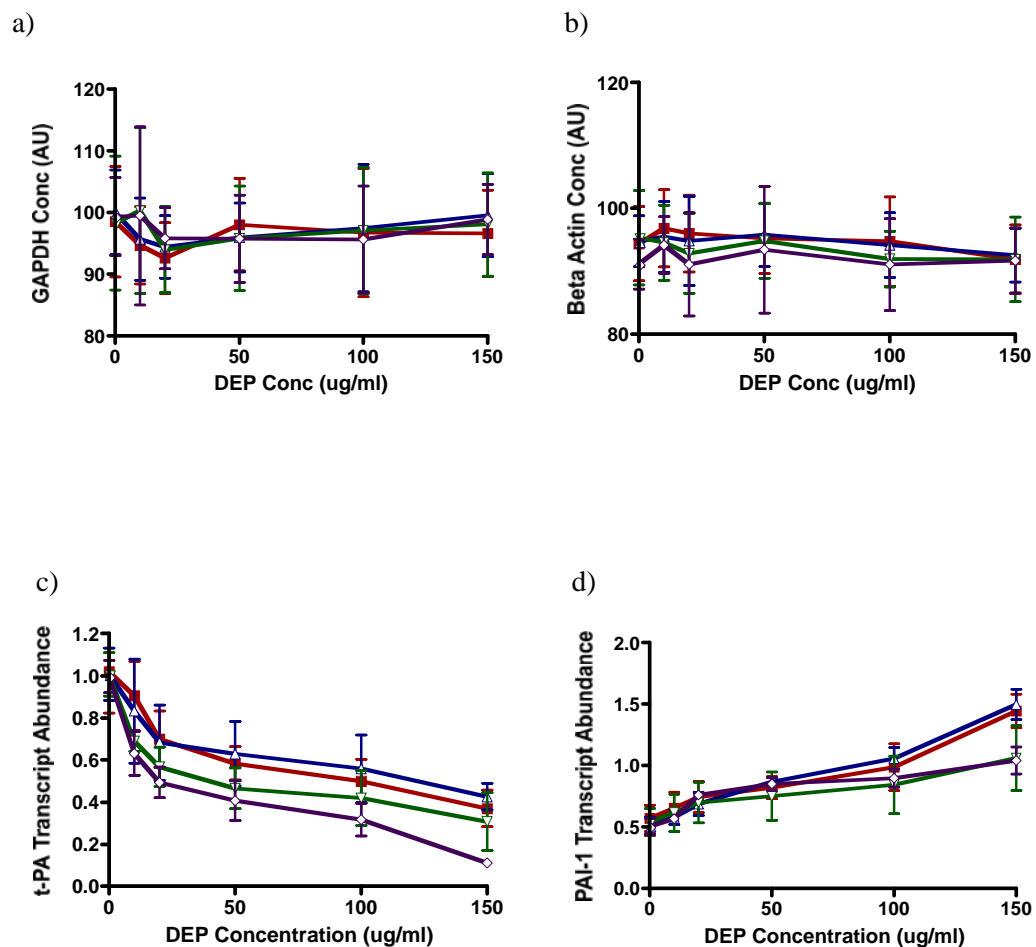
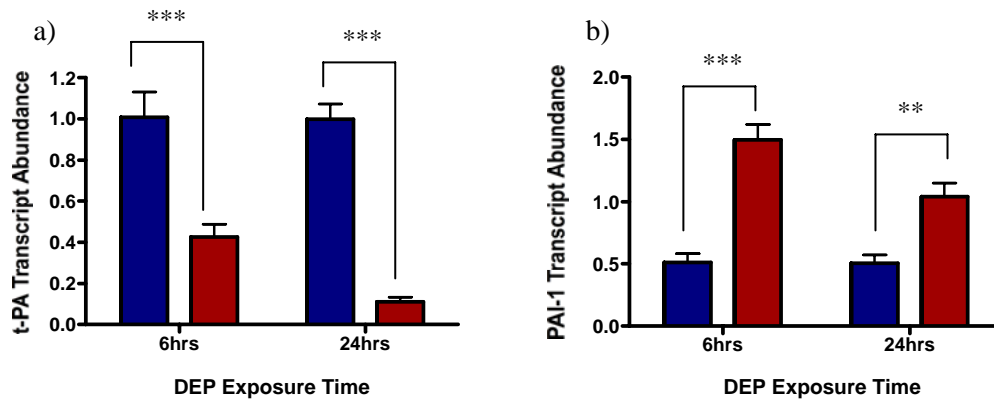


Figure 5.9 - Effects of DEP Exposure on Basal Expression of Fibrinolytic

Factors in Human Endothelial Cells - Final Analysis.

In order to confirm that exposure to diesel exhaust particulate (DEP) reduced expression of t-PA but increased expression of PAI-1 in HUVECs, selected experiments were repeated to allow statistical analysis of results. This confirmed that exposure to DEP (150 μ g/ml; red columns) for 6 or 24 hours significantly reduced t-PA expression (a) but increased PAI-1 expression (b) compared with controls (blue columns). As indicated in preliminary experiments, the increase in PAI-1 expression was larger after 6 hours incubation, than after exposure to DEP for 24 hours. Columns represent mean \pm SEM (n=6). Comparisons were made using Student's unpaired t-test; **P<0.01, ***P<0.001.



5.3.7 DEP-induced Stimulation of the Fibrinolytic System in HUVECs

5.3.7.1 Thrombin-mediated t-PA release from HUVECs - Pilot Investigation

In order to assess the ability of thrombin to stimulate t-PA release HUVECs were exposed to thrombin (0.1-1.0 U/ml) for 6 or 24 hours and t-PA antigen measured in the supernatants by ELISA (t-PA Combi Actibind ELISA kit; Technoclone GmbH, Vienna, Austria). Evidence of t-PA release was observed in unstimulated HUVECs after 24 hours (but not after 6 hours) in culture (Figure 5.10). Thrombin had no effect at 6 hours but produced a concentration-dependent release of t-PA after 24 hours stimulation. Statistical analysis of these data was not performed due to the low group number (n=3). However, on the basis of this preliminary data 24 hour exposure to thrombin was selected for subsequent experiments.

5.3.7.2 Effects of exposure to DEP on t-PA and PAI-1 Expression in HUVECs

Quantitative real-time PCR showed that exposure of HUVECs to thrombin caused an increase in the expression of both t-PA (Figure 5.11c) and PAI-1 (Figure 5.11d). These increases were attenuated by pre-exposure to DEP (16 hours, 100µg/ml). In contrast, expression of controls (GAPDH and β-actin) was unaffected by incubation with thrombin (Figures 5.11a and 5.11b). Statistical analysis of these data was not performed due to small group sizes (n=3). In order to enable statistical comparison, selected conditions (16 hour exposure to 100µg/ml DEP followed by 24 hour incubation with serum free medium or 0.1-1.0U/ml thrombin) were repeated to increase group sizes. This confirmed that pre-incubation with DEP significantly reduced the expression of both t-PA (Figure 5.12a) and PAI-1 (Figure 5.14b) both in unstimulated cells and following stimulation with thrombin.

Figure 5.10 - Thrombin-Mediated Stimulation of t-PA Release from HUVECs.

HUVECS were exposed to thrombin (0,1-1.0U/ml), or vehicle, for 6 (light-grey columns) or 24 (dark-grey columns) hours and t-PA antigen concentration was measured in the supernatant using a commercially available ELISA. In vehicle-treated cells, t-PA was virtually undetectable after 6 hours' incubation but had increased approximately 10 fold after 24 hours. Similarly, thrombin did not increase t-PA secretion after 6 hours but induced a concentration-dependent increase in t-PA antigen after 24 hours exposure. Columns represent mean \pm SEM (n=3). Data were not analysed statistically due to small group sizes.

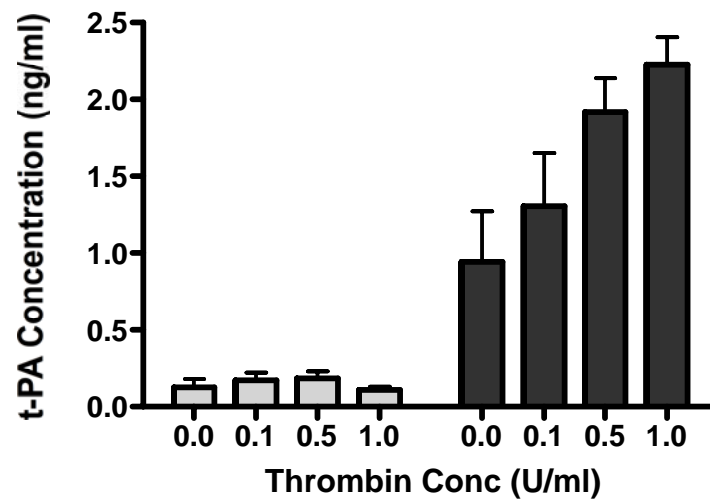


Figure 5.11 - Pre-Incubation with DEP Inhibits Thrombin-Induced Expression of Fibrinolytic Factors in HUVECs.

Human umbilical vein endothelial cells (HUVECs) were exposed to diesel exhaust particulate (DEP; 100 μ g/ml, 16 hours. Blue line) or saline (16 hours. Red line). Cells were then washed and stimulated with thrombin (0.1-1.0U/ml, 24 hours) These treatments had no effect on expression of GAPDH (a) or β -actin (b). Incubation with thrombin produced an apparent increase in expression of tissue-plasminogen activator (t-PA; c) and plasminogen activator inhibitor (PAI-1; d). Furthermore, thrombin produced a concentration-dependent increase in expression of both fibrinolytic factors, which was dramatically attenuated by pre-incubation with DEP. Symbols represent mean \pm SEM (n=3). Statistical analyses were not performed because of small group sizes.

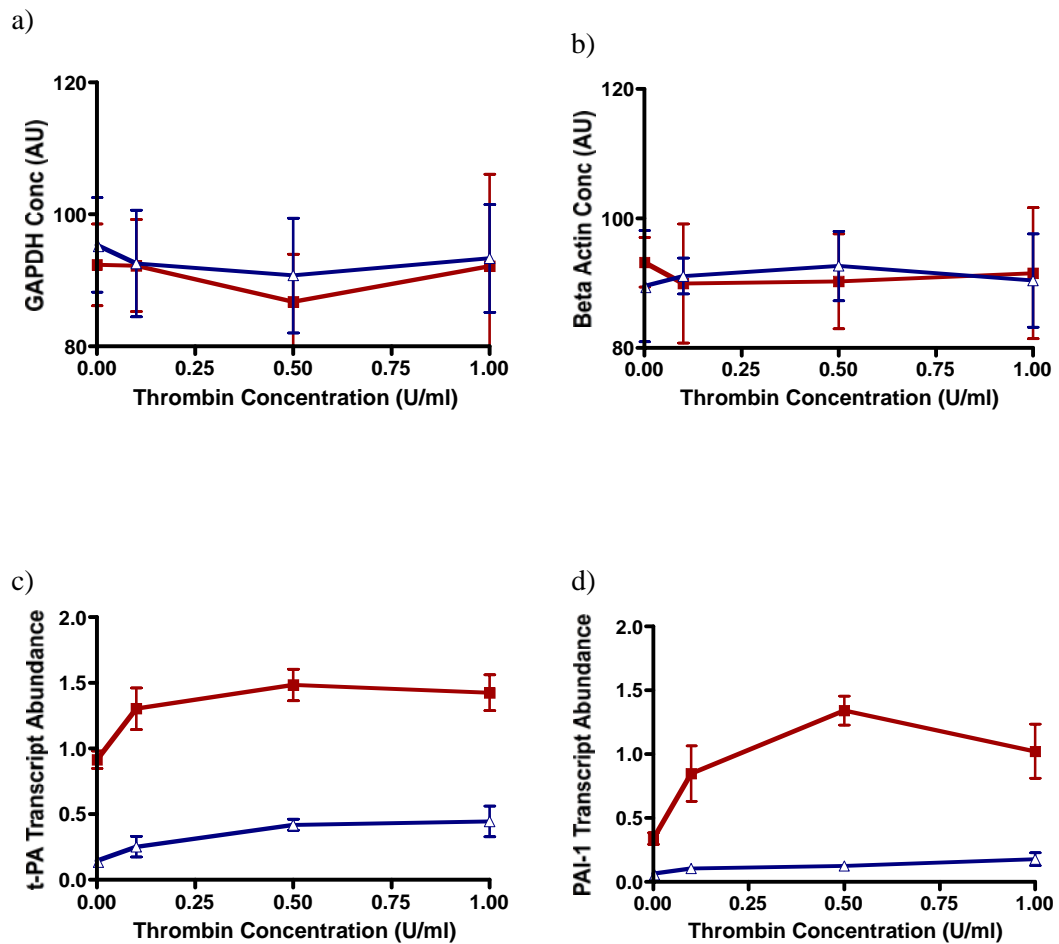
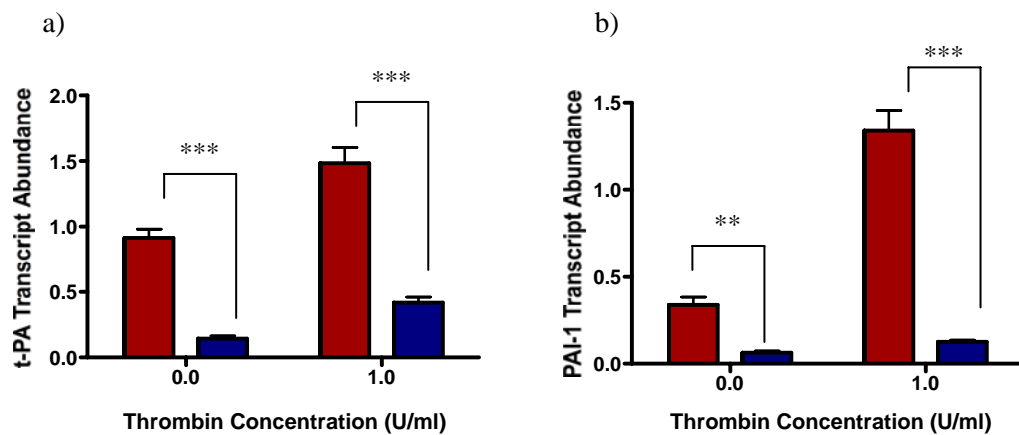


Figure 5.12 - Effects of DEP Exposure on Stimulated Expression of Fibrinolytic Factors in Human Endothelial Cells.

In order to confirm the effects on expression of fibrinolytic factors of pre-incubation with diesel exhaust particulate (DEP; 100 μ g/ml, 16 hours, blue columns) in un-stimulated (red columns) human umbilical vein endothelial cells (HUVECs) and in cells stimulated with thrombin (1.0U/ml 24 hours), selected experiments were repeated to allow statistical analysis. This confirmed that pre-exposure to DEP significantly reduced expression of both tissue plasminogen activator (t-PA) and plasminogen activator inhibitor (PAI-1) in un-stimulated (0.0U/ml thrombin) and in stimulated (1.0U/ml thrombin) cells. Columns represent mean \pm SEM (n=6). Comparisons were made using Student's unpaired t-test; **P<0.01, ***P<0.001.



5.3.8 Effects of DEP Exposure on Basal and Stimulated Secretion of Fibrinolytic Factors by HUVECs

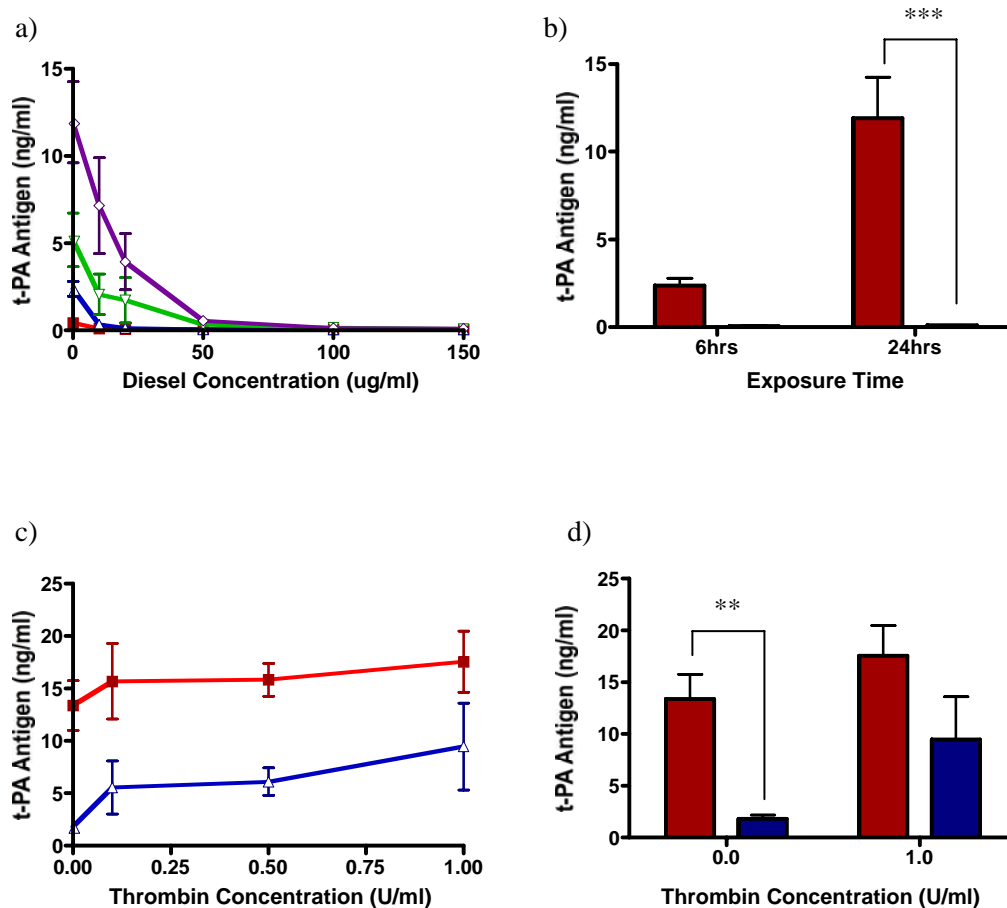
5.3.8.1 The Effects of DEP on Basal and Stimulated t-PA Activity and Antigen Expression

The combined t-PA activity and antigen ELISA failed to produce reliable results for the t-PA activity study (not shown). In vehicle-treated cells t-PA antigen levels increased in a time dependent manner in HUVEC supernatants (Figure 5.13a; n=3). At all time-points (2-24 hours), incubation with DEP (10-150µg/ml) produced a concentration-dependent inhibition of t-PA antigen levels. In order to enable statistical analysis, group numbers were increased for selected conditions (150mg/ml DEP; 6 hours and 24 hours; Figure 5.13b). This confirmed that DEP did significantly reduce constitutive t-PA antigen levels in HUVEC supernatant.

t-PA antigen levels were considerably lower in HUVECs pre-treated with DEP (150mg/ ml, 16 hours) followed by incubation (24 hr) in serum free medium than in those pre-incubated with vehicle (Figure 5.13c). Stimulation with thrombin (0.1-1.0U/ml, 24 hours) produced a small increase in t-PA antigen levels in HUVEC supernatants, with the lowest concentration of thrombin producing almost the maximal response (Figure 5.13c; n=3). Levels of t-PA antigen were dramatically reduced after exposure to DEP, although this appeared to be due to a reduction in basal levels, rather than to an impairment of the thrombin-stimulated release of t-PA. Increasing the group size to allow statistical analysis (Figure 5.13d; n=6) confirmed that t-PA levels were lower reduced by pre-exposure to DEP in un-stimulated cells and in those stimulated with thrombin (0.5U/ml; 24 hours).

Figure 5.13 - The Effects of DEP on Basal and Stimulated t-PA Secretion from HUVECs.

The effects of prolonged culture and exposure to diesel exhaust particulate (DEP) and thrombin on release of tissue plasminogen activator (t-PA) from human umbilical vein endothelial cells were assessed by antigen levels in cell supernatants. (a) t-PA antigen increased in cells exposed to vehicle (0 DEP) in a time-dependent manner. Exposure to DEP (10-150 μ g/ml) produced a concentration-dependent suppression in t-PA antigen levels at all time-points (2hr, red; 6 hr, blue, 16 hr, green; 24 hr, purple; n=3). (b) Increasing group sizes (n=6) confirmed that DEP (150 μ g/ml, blue columns)-induced inhibition of t-PA antigen levels was statistically significant, compared with vehicle (serum-free medium)-treated controls (red columns) after 24 hours in culture. (c) Thrombin (0.1-1.0U/ml) produced a small increase in t-PA antigen levels (n=3). These were reduced in HUVECs pre-incubated with 100 μ g/ml, DEP (red) although this appeared to be due to a reduction in basal levels rather than inhibition of thrombin-stimulated release. (d) Increasing group sizes confirmed that t-PA antigen levels were lower in supernatants from both un-stimulated and thrombin-treated HUVECs after pre-incubation with DEP. Symbols and columns represent mean \pm SEM. Statistical comparisons were made using Student's unpaired t-test **P<0.01, ***P<0.001.



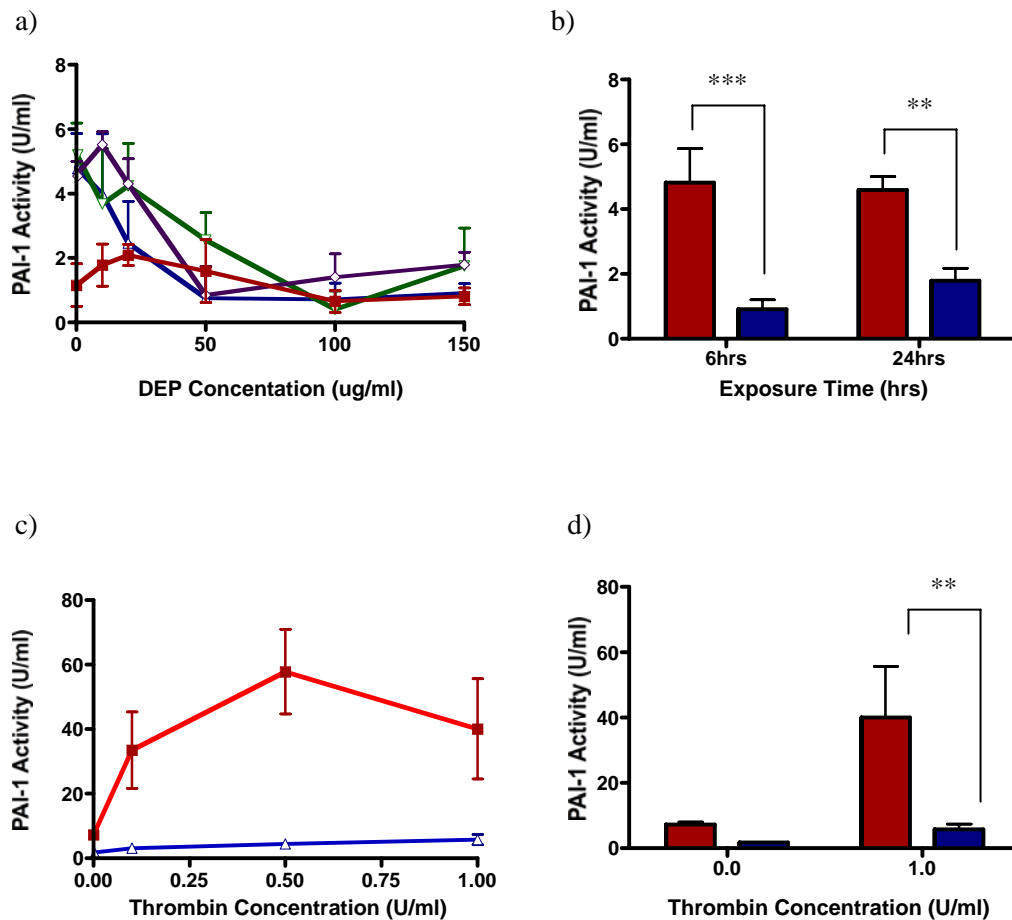
5.3.8.2 The Effects of DEP on Basal and Stimulated PAI-1 Activity

PAI-1 activity in the supernatant of HUVECs cultured (2-24 hours) in the absence of DEP showed an apparent increase over the first 6 hours, but then remained stable up to 24 hours (Figure 5.14a). Exposure to DEP (10-150 μ g/ ml) produced an apparent concentration-dependent reduction in PAI-1 activity at 6, 16 and 24 hour (but not the 2 hr) time-points (n=3). However, increasing group sizes (n=6) for selected conditions (150 μ g/ ml DEP) indicated that levels of PAI-1 activity were similar in DEP- and vehicle-treated HUVECs after incubation for 6 and 24 hours (Figure 5.14b).

Prolonged (16 hr) incubation of HUVECs with DEP (100 μ g/ ml) followed by exposure to serum free medium (24 hr; vehicle control) did not dramatically alter PAI-1 activity in cell supernatants (Figure 5.14c; n=3). However, stimulation with thrombin (0.1-0.5 μ g/ ml; 24 hours) produced an apparent concentration-dependent increase in PAI-1 activity that was abolished by pre-incubation with DEP. Increasing selected group sizes (n=6) confirmed that pre-exposure to DEP attenuated PAI-1 activity in supernatant from thrombin (0.5U/ml; 24 hrs)-stimulated HUVECs but not in vehicle-treated controls (Figure 5.14d).

Figure 5.14 - The Effects of DEP on Basal and Stimulated PAI-1 Activity.

The effects of prolonged culture and exposure to diesel exhaust particulate (DEP) and thrombin on activity of plasminogen activator inhibitor (PAI-1) released from human umbilical vein endothelial cells were assessed enzyme linked immunosorbant assay (ELISA). (a) PAI-1 activity increased over the first 6 hours in cells exposed to vehicle (0 DEP) but then remained stable. Exposure to DEP (10-150 μ g/ ml) produced a concentration-dependent suppression in t-PA antigen levels at all time-points (6 hr, blue; 16 hr, green; 24 hr, purple) except 2hr (red). n=3. (b) Increasing group sizes (n=6) showed that exposure to DEP (150 μ g/ ml, blue columns) reduced PAI-1 activity compared with vehicle (serum-free medium)-treated controls (red columns) after 6 and 24 hours in culture. (c) Thrombin (0.1-1.0U/ml) increased PAI-1 activity levels (n=3) but this effect was virtually abolished in HUVECs pre-incubated with 100 μ g/ml, DEP (red). (d) Increasing group sizes (n=6) confirmed that PAI-1 activity was lower in supernatants from thrombin (0.5U/ml)-treated (but not from un-stimulated) HUVECs after pre-incubation with DEP. Symbols and columns represent mean \pm SEM. Statistical comparisons were made using Student's unpaired t-test **P<0.01, ***P<0.001.



5.4 Discussion

The main hypothesis investigated in this chapter was that direct exposure of endothelial cells to DEP would induce changes in the activity of the endogenous fibrinolytic system. Exposure to suspensions of DEP reduced constitutive expression and antigenic levels of t-PA whilst increasing expression but reducing activity of PAI-1 in unstimulated HUVECs. Stimulation of HUVECs with thrombin caused a concentration-dependent increase in t-PA and PAI-1 expression mirrored by an increase in t-PA antigen and PAI-1 activity. Exposure to DEP prior to stimulation with thrombin reduced expression of both t-PA and PAI-1 and inhibited the elevation of t-PA antigen and PAI-1 activity.

5.4.1 Use of HUVECs to Test the Direct Effects of DEP on the Vascular Endothelium

Cell culture has clear advantages for assessing the direct effects of DEP on the vascular endothelium as it allows controlled exposures under well-defined conditions. Consequently, this approach has been used to assess the effects of various atmospheric pollutants on isolated cell populations, including endothelial cells (Terada *et al.* 1999; Bai *et al.* 2001; Hirano *et al.* 2003; Sumanasekera *et al.* 2007; Andersson *et al.* 2009; Xu *et al.* 2009; Shaw *et al.* 2010), epithelial cells (Terada *et al.* 1999; Matsuo *et al.* 2003) and leukocytes (Hirota *et al.* 2008; Shaw *et al.* 2010). It is possible to generate primary endothelial cell cultures from rats but for these studies it was decided to use an established source of human endothelial cells, as these would be more readily applicable (than rat cells) to the clinical situation. Cultured HUVECs de-differentiate after repeated passaging and lose characteristics of quiescent endothelial cells (Partanen *et al.* 1992). For this reason all HUVECs were used up to passage 6. RT-PCR confirmed that passage 6 HUVECs still expressed the endothelial cell marker Tie-2.

5.4.2 Development of a Suitable Dispersant for Suspension of DEP

Surprisingly few investigations have exposed cultured cells to suspensions of DEP (Furuyama *et al.* 2006) and those studies that are available have used a variety of solutions to suspend DEP (Sagai *et al.* 1993; Ichinose *et al.* 1995; Hashimoto *et al.* 2000; Matsuo *et al.* 2003; Alfaro-Moreno *et al.* 2006). The choice of suspending medium was important as a vehicle was needed that would not compromise cell viability, would enable generation of a range of DEP concentrations, would keep the particles in suspension and would prevent excessive clumping of the nanoparticles. Preliminary investigations demonstrated that serum free EGM-2 was not suitable as a dispersant as DEP did not remain in suspension and measurement of particle diameter suggested clumping. Some studies reporting the effects of DEP on cultured cells do not report how dispersion was achieved (Takizawa *et al.* 2000), while others simply used cell culture medium (Hashimoto *et al.* 2000; Alfaro-Moreno *et al.* 2007) without reporting the resulting particle size or suspension stability. One group used the detergent Tween 80 (0.0025%) to disperse DEP for cell culture experiments (Matsuo *et al.* 2003), without apparent consideration to the effects of the detergent on cell viability. Addition of Tween 20 to EGM produced stable suspensions with particle size in the required range (<200nm). However, pilot data also suggested that Tween 20, even at concentrations of 0.01% in EGM-2, caused significant endothelial cell death (LDH Assay). This was considered most likely due to the ability of detergents to disrupt cell membranes (Culvenor *et al.* 1982). Given the pilot data showing that Tween 20 was toxic to HUVECs, attempts were made to find an alternative dispersant for DEP for use in subsequent experiments.

Since DEP entering the lungs will encounter pulmonary surfactant, it was proposed that modelling this environment might provide a method for maintaining particulates in suspension. This possibility was assessed using a synthetic form of DPPC (a phospholipid that is the strongest surfactant in the lungs; Fisher & Dodia. 1997). Use of this compound was intended simply to improve dispersion of DEP, not to model the intra-pulmonary environment. Initial results were encouraging, as addition of DPPC to EGM-2 helped keep the particulates in suspension and appeared to prevent

clumping. However trials with DPPC were discontinued as others in the group produced evidence to suggest that DPPC was toxic to cultured cells (Dr Craig Poland, Centre for Inflammation Research, University of Edinburgh). This is, perhaps unsurprising, since surfactants (including DPPC) have been shown previously to cause cell lysis (Findlay *et al.* 1995).

Pilot investigations indicated that stable DEP suspensions were formed in 'growth medium' (EGM-2 containing growth factors and foetal calf serum). However, this medium was not suitable for the experiments planned as it stimulates HUVEC proliferation. Healthy ECs *in vivo* are generally quiescent (Zhang *et al.* 1999) and, therefore, a non-proliferative equivalent to growth medium was required. This would also make any exposure results easier to interpret, as cell proliferation would not occur in any exposures. Therefore mRNA expression and protein levels were always calculated from the same number of endothelial cells. To this end, BSA was added to serum free EGM-2, as it is a large protein used to simulate blood composition, and has been used in previous cell culture experiments to stabilise experimental suspensions (Shires *et al.* 1989; Fischer *et al.* 1995). Early investigation found that BSA provided optimal DEP dispersal when it was used at half the concentration of DEP in serum free cell culture medium; a high proportion of DEP was retained in a stable suspension (0.652mg/ml from an original 1mg/ml stock, after sonication and centrifugation) with a low mean particle diameter (<50nm). Consequently, EGM-2 containing 0.5mg/ml BSA was chosen as the dispersant for DEP for use in cell culture experiments. Having identified a suitable dispersant it was then possible to expose HUVECs to DEP suspensions and assess whether this treatment altered the activity of the endogenous fibrinolytic system in these cells.

5.4.3 Does DEP alter activity of the Endogenous Fibrinolytic System in HUVECs?

Few previous studies have reported the effects of exposing endothelial cells to pollutant particulates on the fibrinolytic system. It is apparent, however, that DEP can influence endothelial cell function as much research has been conducted into the

effects of DEP on oxidative stress and DNA damage (reviewed by Risom *et al.* 2005). Using suspensions prepared in EGM-2 plus BSA, HUVECs were exposed to DEP over a range of concentrations (0-150µg/ml) and timepoints (2, 6, 16 and 24 hours) in order to assess its effects on the basal release of fibrinolytic factors. Analysis of the cells using real time PCR showed a decrease in t-PA expression, but an increase in PAI-1 expression, which were both time and concentration dependent. Measurement of antigen levels in the culture medium indicated that t-PA accumulated over time but this was completely abolished in the presence of DEP. PAI-1 activity also increased in medium over time (although this peaked earlier than t-PA antigen; at 6, rather than 24, hours) and was also inhibited in the presence of DEP. This suppression of PAI-1 is consistent with the effects previously reported with organic extracts of DEP and ultrafine particles (Furuyama *et al.* 2006), although Furuyama *et al.* (2006) found no effect of these compounds on t-PA production. Interestingly, though, exposure to carbon black (CB) has been shown to inhibit both t-PA expression and protein production in cultured HUVECs (Gilmour *et al.* 2005). It is also notable, however, that the measurements of mRNA are not consistent with measurements obtained by ELISA. Most striking is the increased PAI-1 expression in response to DEP in the cells but reduced PAI-1 activity in the medium. Since DEP was present in the medium for the course of this experiment, it is possible that t-PA and PAI-1 released into the medium were bound by the DEP. If this is the case, the measurements obtained by ELISA must be viewed with caution. However, the ability of DEP to bind these proteins, or inhibit their activity, would be interesting as this may contribute to the effects of nanoparticulates *in vivo*.

The second series of experiments was designed to investigate the effects of DEP on stimulated release of fibrinolytic factors. To this end, HUVECs were pre-incubated with DEP (or serum free medium alone), followed by stimulation with thrombin (Hanss & Collen. 1987). One bonus of this study design is that DEP is absent from the culture medium during stimulation with thrombin, removing the possibility that fibrinolytic factors may be absorbed by suspended particulate. Thrombin was chosen to stimulate fibrinolytic factor production as it has been shown previously to increase the expression and release of t-PA and PAI-1 from bovine (Fukushima *et al.* 1989)

and human endothelial cells (Gelehrter *et al.* 1986), including HUVECs (Hanss & Collen. 1987; Seljeflot *et al.* 1995). These experiments showed that pre-incubation with DEP caused significant reductions in thrombin-stimulated t-PA and PAI-1 expression and release. In these experiments the results of the ELISAs mirrored the PCR results. Since the DEP in these experiments was washed from the cells before adding thrombin, this supports the proposal that the presence of DEP in the cell culture medium may have affected the results of the original experiment studying basal expression. No previous studies investigating the effects of particles on stimulated release of fibrinolytic factors are available in the literature. Preliminary investigations demonstrated that, after 6 hours in culture, significant concentrations of t-PA could not be detected in medium from unstimulated HUVECs and exposure to thrombin had no effect. In contrast, after 24 hours in culture, t-PA was detectable in the medium and exposure to thrombin produced an apparent concentration-dependent increase in t-PA concentrations. Using these exposure conditions, quantitative real time PCR confirmed that pre-exposure to DEP reduced expression of t-PA in unstimulated cells. Consequently, t-PA expression was lower in thrombin-stimulated cells and the thrombin-induced increase in expression appeared blunted. Measurements of t-PA in the culture medium were broadly consistent with these results. Pre-incubation with DEP reduced t-PA concentration in culture medium, but did not block the thrombin-stimulated release of t-PA. One possible interpretation of these results was that the pre-incubation with DEP reduced the cells' ability to produce or release t-PA; even though the DEP suspension was washed from the cells prior to incubation with thrombin, DEP was observed adhered to the cell surface, which remained in place throughout the thrombin incubations. It is possible that the adhered DEP reduced the cells' ability to react to the thrombin signal, resulting in reduced t-PA mRNA expression and antigen levels. It is also possible that this mechanism was at work in cells exposed to only SFM for the second exposure; reducing resting t-PA mRNA expression and antigen levels.

Measurements of the effects of DEP on thrombin-induced PAI-1 activity produced conflicting data. In contrast with the original experiments (measuring basal PAI-1), pre-incubation with DEP produced a small, but significant, reduction in PAI-1

expression. This contrasts with the apparent increase observed in the previous investigations. As with t-PA, exposure to thrombin produced a concentration-dependent increase in PAI-1 expression in cells and activity in the medium; both these increases were completely abolished in cells previously exposed to DEP. As with the previous experiments, investigating the effects of DEP on thrombin stimulated t-PA mRNA expression and antigen levels, it is possible that DEP adhered to the cell surface reduced the cells ability to react to the thrombin signal in order to produce and release PAI-1.

The DEP-induced changes in t-PA and PAI-1 indicate that direct exposure of endothelial cells to DEP can produce alterations in the endogenous fibrinolytic system both under basal conditions and following stimulation. The reduced expression and release of t-PA is consistent with measurements of plasma t-PA following intravenous injection of DEP (Chapter 4) and also with the previous demonstration that inhalation of DEP alters endogenous fibrinolysis in humans (Mills *et al.* 2005). Interestingly, this clinical study suggested that exposure to DEP did not alter basal circulating levels of t-PA or PAI-1, but did attenuate bradykinin-induced release of t-PA 6 hours (but not 2hrs) after inhalation. The timing of these changes suggests the involvement of an inducible pathway or changes in protein synthesis. The reduced t-PA generation in response to DEP is similar to changes reported in cigarette smokers (Newby *et al.* 1999) and linked to atherothrombosis (Newby *et al.* 2001). Human exposure to DEP has not always produced demonstrable changes in fibrinolytic factors; however, two previous studies indicate no change in a variety of coagulation factors, including t-PA and PAI-1 (Carlsten *et al.* 2007; Baccarelli *et al.* 2007). Combination of these *in vivo* and clinical studies with the data reported in this chapter could indicate a direct effect of DEP on the endothelial-fibrinolytic system. It was notable, however, that injection of CB (Chapter 4) produced a similar reduction in t-PA *in vivo*. This suggests that interaction with endothelial cells is a property common to nanoparticulates and is consistent with previous demonstrations that CB can influence endothelial cell function (Gilmour *et al.* 2005; Yamawaki & Iwai. 2006; Shaw *et al.* 2010). This interpretation might be an over simplification, however, as instillation of particulates

(DEP, CB and quartz) also reduced t-PA in the plasma (Chapter 4). It is conceivable that translocation of particles, and thus direct interaction with the endothelium, could be occurring with DEP and CB (although it seems unlikely that this could produce a sufficiently high concentration of particles in the blood). Quartz, by contrast, is too large to translocate and, thus, the alterations in t-PA activity are more likely secondary to the inflammatory response to particle administration. Chronic inflammation has been associated with impairment of the endogenous fibrinolytic system *in vivo* (Speidl *et al.* 2005; Agirbasli *et al.* 2006; Matejovic *et al.* 2007), while the antioxidant ascorbic acid has been shown to restore the fibrinolytic function of chronic smokers by reducing systemic inflammation (Kaehler *et al.* 2008). Inflammatory stimuli, such as endotoxin, have also been shown to reduce fibrinolytic activity both in animal models (Krishnamurti *et al.* 1991) and in cultured endothelial cells (Colucci *et al.* 1985; Schleef & Loskutoff. 1988; al-Azhary *et al.* 1994). This mechanism is likely to occur in animals exposed to quartz, and to a lesser extent CB and DEP, and may be partly responsible for the changes in expression of fibrinolytic factors observed with all 3 particles.

The effects of DEP on PAI-1 in HUVECs were less consistent and do not fit so readily with the *in vivo* data. Initial demonstrations of increased PAI-1 expression in response to DEP (in unstimulated cells) were consistent with a small increase in circulating PAI-1 following injection or instillation of particulates *in vivo* (Chapter 4). This was not supported, however, by subsequent investigations in which pre-exposure to DEP reduced both unstimulated and basal expression and activity of PAI-1. These differences are difficult to explain and must be attributed to variations in study design. What they do indicate, however, is that direct exposure to DEP can alter PAI-1 expression in HUVECs, even if the nature of this alteration remains unclear. As with the t-PA, changes in PAI-1 *in vivo* were common to all particulates and independent of exposure route. This suggests, therefore, that alteration of PAI-1 may be a feature of nanoparticulates in general (rather than an exclusive property of DEP) and may also be influenced by inflammatory responses *in vivo*.

The results of these cell culture experiments support the hypothesis that DEP can directly alter the endogenous fibrinolytic system in endothelial cells. Thus, if DEP can translocate from the lung to the systemic circulation, it is possible that their actions on the endothelium could contribute to increased thrombus formation. The evidence for particle translocation, however, remains very sparse and was not assessed directly in the work described in this thesis. It should be noted that all of these proof-of-concept experiments used high concentrations of DEP and it seems unlikely that sufficient particles could move from the lungs to alter endothelial cell function; at least in acute investigations. Chronic exposure to DEP could conceivably result in particle translocation and accumulation at vulnerable sites in the vasculature but this remains to be shown. The mechanisms through which DEP interact with HUVECs were not investigated in these experiments but there is considerable evidence to suggest that the toxicity (Donaldson *et al.* 2003) and pro-inflammatory capabilities (Baeza-Squiban *et al.* 1999; Nel *et al.* 2001) of these particles is mediated by oxidative stress. It is also possible that DEP enter endothelial cells via scavenger pathways (Apopa *et al.* 2009). Recent work by my group has shown, using electron microscopy, that DEP accumulate within cultured endothelial cells following exposures similar to those described in this chapter (Shaw *et al.* 2010). If this process also occurs *in vivo* it may provide further insight into the mechanism(s) through which DEP alters the endogenous fibrinolytic pathway in endothelial cells.

Chapter 6

General Discussion

6.1 Summary

The main hypothesis addressed in this thesis is that exposure to air pollution, specifically diesel exhaust particulate (DEP) pollution, increases thrombogenicity through alterations in the endogenous thrombolytic system. There is some limited evidence from clinical and pre-clinical studies to support this hypothesis, and also that nanoparticles in general increase thrombosis and that particulate pollution alters function of the cardiovascular system (Nemmar *et al.* 2003c; Nemmar *et al.* 2004a; Mills *et al.* 2005; Nemmar *et al.* 2005; Radomski *et al.* 2005; Mills *et al.* 2007; Tornqvist *et al.* 2007; Geys *et al.* 2008; Lucking *et al.* 2008; Nemmar *et al.* 2009). To address this hypothesis, it was first necessary to develop a usable experimental model of arterial thrombosis *in vivo* and then to use this model to investigate the effects of DEP on thrombus formation and breakdown. With this model, and the use of control particulates, it was possible to address the importance of systemic and pulmonary inflammation in the modulation of thrombogenesis, by comparing the effects of intravenous and intratracheal administration of particles. This design also provided indirect evidence of the potential role of particle translocation in mediating the response to DEP. Having demonstrated accelerated thrombus formation in response to DEP administration *in vivo*, the second aim was to determine the impact of direct exposure of vascular cells to DEP on the endogenous thrombolytic system, by detailed mechanistic examination of cultured endothelial cells exposed to DEP *in vitro*.

The key results of these studies were that DEP administration, via either instillation or injection, caused an increase in the rate of thrombus formation, which was not dependent on pulmonary or systemic inflammation. This action appeared to be mediated through increases in platelet-monocyte aggregation. Changes in the endogenous fibrinolytic system (reduced tissue plasminogen activator (t-PA) and a tendency to increased plasminogen activator inhibitor (PAI-1)) were detected but were also produced by control particles (which did not alter thrombus formation). *In vitro* investigations confirmed that DEP could alter endogenous fibrinolytic function by direct interaction with endothelial cells.

6.2 Main Conclusions

Several methods were considered for assessing the effects of particulates on clotting and thrombus formation. In initial investigations, measurement of tail tip bleeding time was used to examine blood clotting; with a significant reduction in clotting time observed after intravenous injection of DEP. However, extension of this method beyond the pilot investigations did not replicate these initial results. The only difference between these experiments was the length of time the animals were exposed to anaesthesia; in pilot experiments the animals were sedated for the entire 2 hour exposure period, whereas in the subsequent experiments the animals were allowed to regain consciousness after exposure until the required timepoint for assessment of clotting. This suggests that the length of anaesthesia had an effect on bleeding time in rats, although there appears to be no data available in the literature assessing the effect of extended anaesthesia on clotting activity. It is also possible that the extended period of anaesthesia in preliminary investigations caused a stress response in the experimental animals, leading to increased coagulability, although this cannot be substantiated.

Initial work developing the Folts model of arterial thrombosis proved to be problematic. This model did not reliably cause thrombus formation in experimental animals, and when thrombus formation did occur it was very difficult to embolise; a key step in the quantification of coagulation in this model. Communication with other groups who had previously used this method indicated that poor reliability of thrombus formation and embolisation was a recurrent issue. Therefore, it was decided that an alternative approach should be attempted. The model of ferric chloride (FeCl_3)-induced carotid artery thrombosis proved to be a reliable alternative; consistently generating clot formation in a reproducible time frame. In this model loss of blood flow is measured during thrombus formation at a site of iron-induced injury. Following pilot experiments to establish reproducibility, this model was used to assess the effects of DEP on thrombus formation and inflammation 2, 6 and 24 hours after intratracheal instillation or intravenous injection. These experiments were expanded by the use of control particles; the effects of carbon black (CB) and quartz (DQ12) were examined 6 hours after intratracheal instillation and the effects of CB were

examined 2 hours after intravenous injection. CB is a carbon particle of comparable size to DEP, without the volatile groups, metals and organic compounds associated with the surface of DEP. DQ12 is a large non-carbon particle, which induces pulmonary inflammation without the possibility of translocation into the circulation. Both CB and DQ12 were used as controls in instillation experiments. In contrast, CB alone was used as a control for intravenous administration of DEP, as DQ12 is too large to translocate from the lungs, and was used specifically to investigate the effects of pulmonary inflammation on thrombus formation. These experiments showed that DEP caused a significant reduction in time to thrombus formation, results that were supported by the demonstration of increased plasma levels of fibrinogen and D-Dimer in DEP-treated animals. Lesser reductions in time to thrombus formation were observed with control particles, with statistical significance only observed 2 hours after CB injection. The combination of these results shows that DEP has greater effects on increasing thrombus formation than CB, a clean carbon nanoparticle. This suggests that the volatile groups, metals and organic compounds associated with the DEP surface may play a role in enhancing the effects of the carbon core in terms of thrombus formation. Some compounds associated with the DEP surface, such as hydrocarbons, have been linked to the pulmonary inflammatory actions of DEP (Fahy *et al.* 2009; Holder *et al.* 2007), and possibly cardiovascular actions, including oxidative stress (Hirano *et al.* 2003). However, this is the first time that clear evidence has been provided for the direct role of DEP surface constituents on thrombus formation.

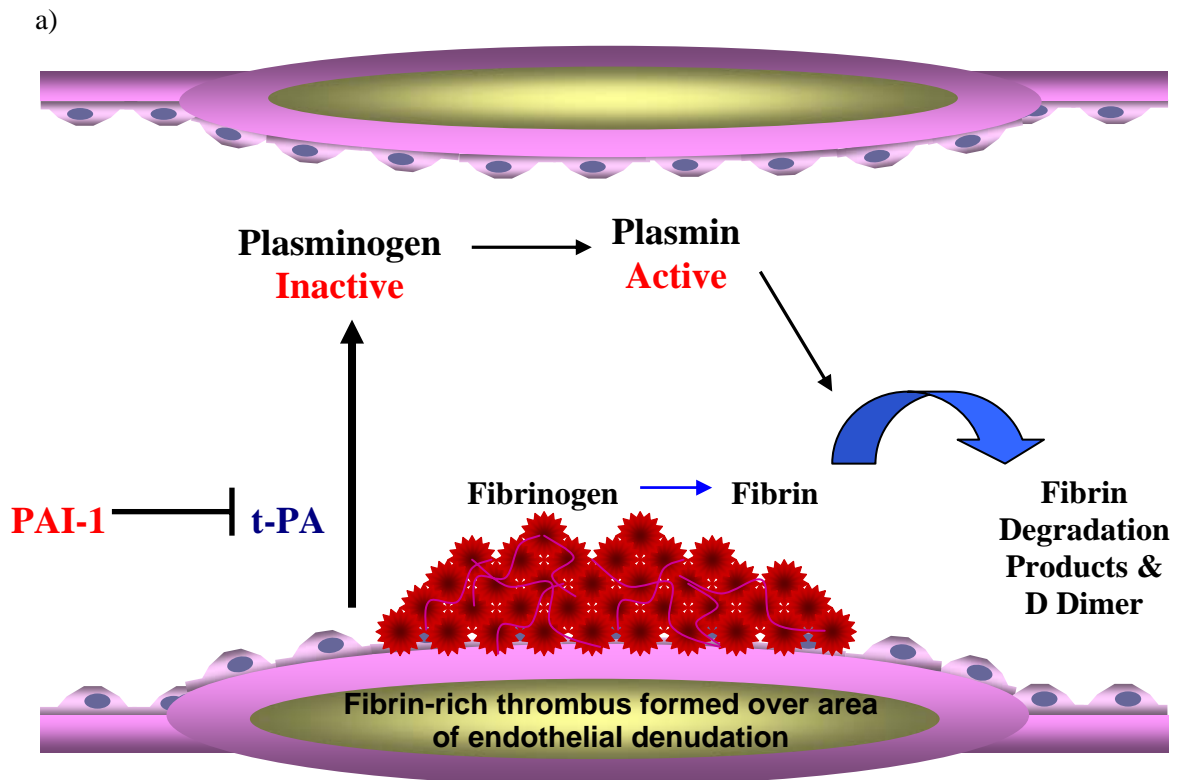
The ability of DEP and control particles to induce pulmonary and systemic inflammation were assessed by measuring their effects on levels of established markers of inflammation; namely, a range of cytokines, including interleukin 6 (IL-6), tumour necrosis factor alpha (TNF α) and C reactive protein (CRP). These cytokines have been used previously as markers of pulmonary and systemic inflammation (Donaldson *et al.* 2001; Saber *et al.* 2006; Sunil *et al.* 2009; Nemmar *et al.* 2010; Mills *et al.*, 2005). These experiments showed that the control particles, CB and DQ12, caused a higher degree of both pulmonary and systemic inflammation than DEP. This was not initially expected; the concentration of DQ12 was chosen specifically to elicit the same inflammatory response as the chosen DEP

concentration. At the start of these investigations it had been anticipated that CB would essentially be an inert particle with few physiological effects. This proved not to be the case, however, as (similarly to DQ12) the level of pulmonary inflammation caused by CB was far in excess of that produced by DEP. This indication that CB is an active particle is consistent with results reported by other groups (Yamawaki & Iwai, 2006) and has been confirmed by recent experiments in our laboratories (Miller *et al.* 2009; Shaw *et al.* 2010), which have shown that CB produces higher levels of free radicals than DEP and are able to stimulate cytokine production from cultured endothelial and inflammatory cells. Comparison of measurements of inflammation and arterial thrombus formation indicated that inflammation cannot be the only factor in determining thrombogenicity. For example, exposure to DEP clearly increased thrombus formation but had relatively small effects on systemic and pulmonary inflammation. In contrast, CB and DQ12 caused considerable pro-inflammatory changes but had relatively modest (if any) effects on thrombogenesis.

In order to further understand the mechanisms through which DEP accelerated thrombus formation, a series of assays were performed to assess the effects of nanoparticles on platelet function and the fibrinolytic system. DEP injection and instillation caused increases in platelet-monocyte aggregation, consistent with previous demonstrations that platelet activation is involved with the increase in thrombogenicity (Lucking *et al.*, 2008; Nemmar *et al.*, 2003). DEP also reduced protein levels of t-PA, while control particles appeared to have more of an impact on increasing PAI-1 protein levels. Regardless, when the t-PA/PAI-1 ratio was examined, all three particles produced a similar reduction when compared with saline controls. It is clear that DEP has an impact on t-PA and PAI-1 expression, but these effects alone do not explain the differences in thrombus formation observed between DEP and the control particles. An outline of the fibrinolytic system is given, and the changes in this system caused by particle exposure outlined, in Figure 6.1.

Figure 6.1 - Changes in the Fibrinolytic System Caused by Particulate Exposure.

a) Outline of the fibrinolytic system. Thrombus formation in the vessel is generated by platelet activation and aggregation at the site of endothelial denudation, which is stabilised via the conversion of fibrinogen to fibrin. Endothelial cells release tissue plasminogen activator (t-PA) in response to thrombus formation, which converts inactive plasminogen to active plasmin, which then dissolves the thrombus by breaking down fibrin into fibrin degradation products and D dimer. The actions of t-PA are controlled by plasminogen activator inhibitor (PAI-1), which regulates fibrinolytic activity. b) Table outlining the effects of diesel exhaust particulates (DEP), and control particles carbon black (CB) and quartz (DQ12), on components of the fibrinolytic system. ↑ = increased expression, ↓ = decreased expression, N/C = no change.



b)

	t-PA	PAI-1	t-PA/PAI-1	Fibrinogen	D Dimer
DEP	↓	↑	↓	↑	↑
CB/DQ12	↓	↑	↓	N/C	N/C

In vitro investigations were performed to determine whether the ability of DEP to alter endogenous fibrinolysis, as demonstrated in clinical studies (Mills *et al.* 2005, Mills *et al.* 2007), could be attributed to direct interaction with the vascular endothelium. Much of the initial *in vitro* work involved development of a suitable dispersant for DEP for use in cell culture experiments. This is an issue that appeared not to have been addressed previously in studies investigating the effects of DEP in cell culture. Assessment of the effects of a range of DEP dispersants on particle size and suspension stability found that EGM-2 culture medium containing 0.5mg/ml bovine serum albumin (BSA) was the best dispersant to use in cell culture. Using this dispersant, human endothelial cells were exposed to suspensions of DEP to examine its effects on the basal and stimulated release of t-PA and PAI-1. The results of the experiments on basal release were consistent with the *in vivo* experiments examining the effects of particulate exposure on t-PA and PAI-1; t-PA mRNA transcripts and antigen levels were reduced with increasing DEP exposure, while PAI-1 mRNA transcripts and antigen levels increased. This observation confirms the results of the *in vivo* studies, but also shows that the effects of DEP on components of the fibrinolytic system are both concentration- and time-dependent. Stimulated t-PA and PAI-1 release was examined by exposing endothelial cells to thrombin after incubation of the cells with DEP. This provided an *in vitro* approximation of the processes that accompany thrombus formation *in vivo*, in which an acute release of t-PA is necessary for thrombus breakdown. This exposure reduced expression and secretion of both factors following DEP treatment. The mechanism(s) responsible for this action of DEP was not investigated, although previous investigations implicate a role for oxidative stress. DEP particles were observed to adhere to the endothelial cell surface in culture and it is possible that this may have interfered with the interaction of thrombin with the endothelial cell surface. It seems unlikely that a similar mechanism would be significant in humans exposed, as there is no evidence that sufficient concentrations of DEP translocate from the lungs to the systemic circulation.

In conclusion, the work presented in this thesis examines the effect of DEP exposure on thrombogenicity, pulmonary and systemic inflammation, platelet function and the fibrinolytic system. The results obtained with DEP have been compared with control particles, in order to clarify the mechanisms of the effects observed. Similarly, two different methods of administration were used to determine whether pulmonary

inflammation was necessary to induce the pro-thrombotic effects of DEP. Administration of DEP via the tail vein demonstrated that direct interaction of these particles with the cardiovascular system could alter thrombus formation. Despite this, the contribution of particle translocation to accelerated thrombosis following instillation induced was not examined and remains unresolved. No data were generated to support a role for particle translocation into the systemic circulation after instillation. It is more likely that the inflammatory effects of the three particles investigated have some downstream effects on fibrinolysis, but this does not explain the additional effects of DEP, which caused least inflammation but most pro-thrombotic effects. Comparing the effects of DEP to CB, it seems likely that the volatiles, metals and organic compounds associated with the DEP surface cause further changes to platelet function and the fibrinolytic system, possibly by dissociating from the particle surface in the lungs and translocating into the bloodstream alone.

6.3 Implications

6.3.1 Atmospheric pollution

The demonstration that exposure to DEP accelerates thrombosis is consistent with observations made in clinical studies (Mills *et al.* 2005; Mills *et al.* 2007; Lucking *et al.* 2008). These results are, therefore, applicable to man and may help to influence current health policies and advice designed to inform people about, and protect them from, dangerous exposures to atmospheric pollution. This may be especially important to those at risk of myocardial infarction. The current level of concern about the health implications of atmospheric pollution is emphasised by the recent publication of a report by the Environmental Audit Committee (UK Parliament investigation) and coverage of the issue by the National media (including the BBC).

The most obvious advice suggested by the results of this work would be to avoid sources of significant particulate pollution, particularly emissions from road traffic. However, whilst logical, this advice may simply not be practical, or even possible, for many people to follow in today's society. An alternative approach to reducing

exposure to DEP is through the development of vehicles designed to run using hydrocarbon-free fuels, such as electricity and hydrogen fuel cells, as found in the new Honda FCX Clarity (currently under consumer testing in America). It could be argued that provision of greater resources to develop this type of technology would have clear health-care benefits. Similar arguments would support the further development of bio-fuels and filters designed to reduce particulate emissions from current engine exhausts. Worldwide adoption of vehicular modifications such as these could have a dramatic effect on reducing morbidity and mortality due to cardiovascular events in the developed world, especially in densely populated areas.

In addition to traffic-derived particulate emissions, other sources of particulate pollution, such as wood smoke and coal-fuelled power plants should also be considered. Coal fly-ash from power plants acutely increases pulmonary and systemic inflammation in rats (Smith *et al.* 2006) whilst similar effects of wood smoke have been identified in fire fighters (Swiston *et al.* 2008). These sources of particulate pollution have not, however, been linked with cardiovascular and thrombotic complications, supporting the contention that it is nanoparticles (particularly those such as DEP with combustion-derived compounds on their surface), that have the most dramatic influence on the cardiovascular system.

This work also suggests possible new treatment advice for patients presenting with myocardial infarction. Thrombolysis (using streptokinase, urokinase or t-PA) is currently the standard pharmacological approach to restoring vessel patency following myocardial infarction or stroke (Shaughnessy & Scow. 1996; Rentrop *et al.* 1979). However, strict guidelines must be adhered to before treating a patient with t-PA, as bleeding complications can occur. For myocardial infarction, physical intervention, such as angioplasty (Fung *et al.* 1986), is used to restore patency in individuals for whom thrombolytic therapy is contraindicated. Anti-platelet regimens (such as daily administration of aspirin) can also be used as these improve survival and also enhance the effect of thrombolytic therapy. The data described in this thesis, combined with results of clinical investigations (Mills *et al.* 2005; Mills *et al.* 2007; Lucking *et al.* 2008) suggest that prophylactic anti-platelet therapy (for example with aspirin) may be beneficial in individuals with a high risk of myocardial infarction who are regularly exposed to high levels of air pollution (such as road traffic exhaust). The

benefit of this approach would, obviously, have to be balanced against potential side effects (such as the development of stomach ulcers). Further clinical studies would be required to confirm the benefit of this approach in at-risk patients.

6.3.2 Engineered and Medical Nanoparticles

The work described in this thesis may also have relevance beyond the issue of atmospheric pollution. A clear example of this is in its application to understanding the potential cardiovascular risk of nanoparticle use in clinical practice. Several different types of nanoparticulate (such as quantum dots and ultrasmall paramagnetic iron oxides (USPIOs)) have potential clinical applications; for example for the identification and targeting of cancer cells and for imaging arterial inflammation/lesion development. Quantum dots, nanoparticle crystal semiconductors that emit visible light upon excitation, can be bio-engineered to specifically target cellular markers of disease. Their use is currently being researched to aid identification of pancreatic cancer cells (by conjugation with specific antibodies such as anti-claudin 4 and anti-prostate stem cell antigen (Yong *et al.* 2009)), and breast cancer cells (by conjugation with human epidermal growth factor receptor 2 (Chen *et al.* 2009)). Since quantum dots are much more efficient than traditional organic dyes (they emit a brighter signal and are much more stable (Walling *et al.* 2009)), their use promises to greatly improve our understanding of the mechanisms underlying disease pathogenesis. Current use has been largely limited to *in vitro* testing, but initial attempts have been made to target tumour cells *in vivo* using animal models (Ballou *et al.* 2004). Adverse effects identified using *in vitro* techniques may limit the potential of quantum dots. For example, exposure of quantum dots to ultraviolet light caused the release of (highly toxic) cadmium (Lee *et al.* 2009), although this seems more likely to produce a waste disposal problem than to happen *in vivo*. Previous work in mice has also shown that quantum dots remain in the body for up to 4 months (Ballou *et al.* 2004), indicating a need for long-term studies to ensure that these nanoparticles do not cause vascular injury and inflammation for a significant period of time after administration. This could be important since several different types of nanoparticle (including polystyrene and carbon nanotubes) have detrimental effects on the cardiovascular system; for example, carbon nanotubes accelerate the progression of

atherosclerosis in ApoE knockout mice (Li *et al.* 2007). Given the results reported in this thesis, it must also be considered possible that quantum dots may influence thrombosis. Indeed, one study has shown that high doses of quantum dots cause pulmonary vascular thrombosis, possibly by stimulating the coagulation cascade via contact activation (Geys *et al.* 2008). Similar concerns could be raised for the use of USPIOs, which have potential clinical application for enhancement of molecular resonance imaging of sites of vascular inflammation/lesion formation (Tang *et al.* 2009). Much more work is required to ensure that nanoparticles such as quantum dots and USPIOs do not have the same effects on the thrombolytic system as the DEP described in this thesis.

Another use for engineered nanoparticles is in water purification, with nanoparticulate gold and palladium added to drinking water in order to degrade biological and chemical toxins (Heck *et al.* 2009). Previous work, including data presented in this thesis, shows that the issue of nanoparticle translocation has not been successfully resolved. Although the lungs and the gastrointestinal tract are anatomically very different, it is not inconceivable that a proportion of nanoparticles could enter the blood stream via absorption through the gut and cause changes in the cardiovascular system. Gold nanoparticles are also being developed to target cancer cells, allowing them to be destroyed using a laser, or preventing them from multiplying in areas that cannot be targeted by laser (Kang *et al.* 2010). Again, as with the previous examples, this appears to be an exciting area of research, which has the potential to save many lives. There is no direct evidence linking nanoparticulate gold to cardiovascular disease, and gold is a very inert particle. However, once again the long-term impact of these nanoparticles appears not to have been taken fully into consideration. Further work must be conducted before these treatments are routinely used, in order to fully understand the consequences of exposing people to nanoparticulates.

6.4 Future Work

This work described in this thesis has provided insights into the mechanisms contributing to the reported increase in cardiovascular events following acute exposure to atmospheric pollution. Despite this, many questions concerning the relationship between atmospheric particulate pollution and atherothrombosis remain.

The studies described were designed specifically to investigate the causes of increased cardiovascular events in response to recent exposure to environmental pollution (Peters *et al.* 2004). They were not, however, designed to address the cardiovascular implications of chronic exposure to elevated levels of DEP. There is increasing evidence that chronic exposure to atmospheric pollutants, and particularly to vehicle exhaust emissions, has detrimental effects on the cardiovascular system (for example, see Sun *et al.* 2005). Furthermore, it seems logical that exposure to atmospheric pollution may have similarities to the effects of cigarette smoking, which has well-described cardiovascular consequences (Davies *et al.* 1997; Chen *et al.* 1995) including impairment of endogenous fibrinolysis (Newby *et al.* 1999). If this is true, it is also possible that individuals exposed to atmospheric pollution may exhibit an equivalent of the ‘smokers’ paradox’; that is they may be more likely to develop occlusive thrombotic disease but also may be more responsive to thrombolytic therapy (Purcell *et al.* 1999; Newby & Boon. 1999). These possibilities emphasise the importance of investigating the effects of chronic exposure to DEP on thrombogenesis and the endogenous fibrinolytic system *in vivo*. This could be achieved by repeated instillations or injections of DEP at relevant concentrations over a period of weeks or months. Administration of DEP by inhalation would be desirable in such investigations but would have to be performed carefully to ensure the specified dose of DEP reached the lungs (the complex structure of the rodent nasal passage, and the fact that particles often stick to the coat, may dramatically reduce the pulmonary exposure to atmospheric pollutants).

It would also be interesting to assess the impact of DEP administration on thrombogenesis in animals with pre-existing cardiovascular disease. The effects of acute exposure to atmospheric pollution on cardiovascular events are likely to occur

in individuals with pre-existing atherosclerotic disease. Therefore, it would be logical to apply the FeCl_3 model of arterial thrombosis to animals with pre-existing atherosclerotic lesions and/or endothelial cell dysfunction.

Further experiments could also be performed in an effort to identify more clearly the component(s) of the DEP responsible for the observed changes in thrombogenesis and the endogenous fibrinolytic system. 'Washing' DEP with dichloromethane allows removal of organic compounds from the surface of the particles (Takano *et al.* 2007). The organic compounds and washed DEP could then be tested separately, via instillation and injection using the *in vivo* model described in this thesis, to determine which component(s) contribute to alterations in clotting activity. This approach is currently being used by my group to determine which components of DEP contribute to particulate-mediated alterations in arterial function *in vitro* (Miller *et al.* 2009).

Finally, further study is required for complete clarification of the mechanism responsible for DEP-induced acceleration of thrombus formation. While differences in expression and activity of enzymes within the thrombolytic system were demonstrated, further investigations could be performed to clarify the role of particular components of the endogenous fibrinolytic system in. The use of transgenic animals, such as the PAI-1 deficient transgenic mouse (Liu *et al.* 2009), could help to further dissect the role of the fibrinolytic system following exposure to DEP. Early work described in this thesis found that applying arterial thrombosis models to mice was extremely problematic. However, other groups have reported positive results when using thrombosis models, including the FeCl_3 (Farrehi *et al.* 1998), Rose Bengal (Eitzman *et al.* 2000) and Folts (Sturgeon *et al.* 2006) models in mice. Also, using these models in combination with pharmacological treatments (for example aspirin to reduce platelet activation) would help to improve our understanding of the mechanisms at work, and may advance development of practical treatments to prevent thrombus formation due to particulate pollution exposure.

6.5 Concluding Remarks

The work described in this thesis addressed the hypothesis that acute exposure to DEP accelerates thrombosis by direct modulation of the endogenous fibrinolytic system. Optimisation of an *in vivo* model of thrombosis enabled the demonstration that DEP did indeed increase thrombus formation, without the need for systemic or pulmonary inflammation. Changes contributing to this effect included increased platelet activation and alterations in the endogenous fibrinolytic system. *In vitro* investigations suggested that the latter could have been caused by direct interaction of DEP with the endothelium. Possible future investigations could address the effect of chronic exposure to DEP on disease free animals, on animals with pre-existing cardiovascular disease and on animals with relevant genetic modification. In addition, pharmacological interventions could be applied to animals following (chronic or acute) exposures to both further understand the mechanism responsible for accelerated thrombosis and to identify potential remedies for DEP-induced acceleration of thrombosis. The work could also be extended to clinical trials to examine whether the same mechanisms are at work in humans. Expanding the work in this thesis, as suggested, may promote further testing of the thrombogenic potential of other nanoparticles (such as quantum dots and nanoparticulate gold) and may help inform healthcare policy regarding nanoparticulate exposure. Overall, this study has provided more evidence that exposure to DEP promotes thrombus formation, and has identified key components of the thrombotic and fibrinolytic systems that are responsible for these effects. The results presented in this thesis suggest that reducing, or removing, particulate pollution may significantly reduce cardiovascular morbidity and mortality.

Chapter 7

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